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Role of TRAP1 in the adaptive metabolic response under hypoxic conditions in human colorectal carcinoma

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ABSTRACT

Energy metabolism reprogramming, which fuels fast cell growth and proliferation by adjustments of energetic resources, is considered as an emerging hallmark of cancer. The common feature of this altered metabolism of cancer cells is increased glucose uptake and fermentation of glucose to lactate. This phenomenon is observed even in the presence of oxygen and completely functioning mitochondria. Moreover, solid malignant tumors contain normally hypoxic areas characterized by low levels of oxygen, and this results in a substantial reprogramming of the expression of number of genes involved in cellular metabolism, a process mostly driven by HIF1 alpha. TRAP1 is a HSP90 molecular chaperone upregulated in human colorectal carcinomas (CRCs) and responsible for suppression of oxidative phosphorylation (OXPHOS) and adaptation to stress. In order to characterize the role of TRAP1 in glycolytic metabolism and adaptive metabolic response under hypoxic conditions, patients-derived CRC organoids, human CRC cells and CRC samples were used. A linear correlation was observed between TRAP1 levels and ^{18}F-Fluoro-2-Deoxy-Glucose (^{18}F-FDG) uptake upon PET scan or GLUT1 expression in human CRCs. Consistently, TRAP1 enhances GLUT1 expression, glucose uptake and lactate production and downregulates OXPHOS in CRC cell lines, and this condition was more pronounced under hypoxia, which by itself favors glycolytic metabolism. Interestingly, TRAP1 is involved in regulating hypoxia-induced HIF1alpha stabilization, being its expression partially impaired in TRAP1-silenced CRC cells exposed to hypoxia. Consistently, at transcriptional level, the reprogramming of cancer metabolism driven by HIF1α is partially blocked when TRAP1 is downregulated, with a reduction in expression levels of all transcripts induced by long-term exposure to hypoxia in both HCT116 cells and patients-derived CRC organoids. These data allow us to hypothesize that TRAP1 expression level in colorectal cancer is a key factor for the activation of the oncogenic HIF-1α genetic program, widening the panorama of TRAP1-mediated adaptive responses

in carcinoma of the colorectal. Thus, this concept supports the hypothesis that TRAP1 targeting could constitute a new antitumor approach.

1 INTRODUCTION: GENERAL INFORMATION ON COLORECTAL CANCER

1.1 Definition

Cancer that develops in the colon (the longest part of the large intestine) and/or the rectum (the last several inches of the large intestine before the anus).

1.2 Epidemiology

Colorectal cancer is the second- and third-most common cancer in women and men, respectively. In 2012, 614,000 women (9.2% of all new cancer cases) and 746,000 men (10.0% of new cancer cases) were diagnosed with colorectal cancer worldwide (Kuipers, et al., 2016). Combined, in both sexes, colorectal cancer is the third-most common cancer and accounts for 9.7% of all cancers and more than half of the cases occur in more-developed regions of world. The highest incidence is reported in countries of Europe, North America, and Oceania, whereas incidence is lowest in some countries of south and central Asia and Africa (Ferlay J, 2010).

Incidence - In Italy approximately 51,000 new colorectal cancer diagnoses are estimated in 2018. According to AIRTUM data, both among men (15% of all new tumors) and women (13%) is in second place, preceded respectively by prostate and breast. **Table 1**

Rank	Male	Females	All population
1°	Prostate (18%)	Breast (29%)	Breast (14%)
2°	Colorectal (15%)	Colorectal (13%)	Colorectal (14%)
3°	Lungs (14%)	Lungs (8%)	Lungs (11%)
4°	Bladder * (11%)	Thyroid (6%)	Prostate (9%)
5°	Liver (5%)	Uterus body (5%)	Bladder * (7%)

Table 1 – First five most frequently diagnosed tumors and proportion of total tumors (excluded skin cancers) by sex*. Estimated for Italy 2018 * Includes both infiltrating and non-infiltrating tumors.*

In the classification of the most frequent tumors for age groups, colorectal cancer always occupies high positions, varying in different ages between 7% and 14% in men and between 4% and 16% in women. **Table 2**

Rank	Male (age)			Females (age)		
	0-49	50-69	70+	0-49	50-69	70+
1°	Testicle (12%)	Prostate (22%)	Prostate (19%)	Breast (41%)	Breast (35%)	Breast (22%)
2°	Skin(melanomas) (9%)	Lungs (14%)	Lungs (17%)	Thyroid (15%)	Colorectal (11%)	Colorectal (16%)
3°	Thyroid (8%)	Colorectal (12%)	Colorectal (14%)	Skin(melanomas) (9%)	Lungs (7%)	Lungs (8%)
4°	LNH (7%)	Bladder* (11%)	Bladder* (12%)	Colorectal (4%)	Uterus body (7%)	Pancreas (6%)
5°	Colorectal (7%)	Upper aerodigestive routers**(5%)	Stomach (5%)	Uterus Cervix (4%)	Thyroid (5%)	Stomach (5%)

Table 2 – First five cancers in terms of frequency and proportion of total incident tumors (excluding skin carcinomas) by gender and age group. *Pool AIRTUM 2010-2014. ** Includes both infiltrating and non-infiltrating tumors. ** Includes tongue, mouth, oropharynx, nasopharynx, hypopharynx, pharynx NAS, larynx.*

The spread of risk factors, diagnostic anticipation and the increase in the average age of the population are at the basis of the progressive growth of the incidence of this tumor in past years. The time trend for men has gone from a stable trend until the mid-2000s to a reduction in the 2007-2010 period (-4.0% / year), with a subsequent stabilization, effects associated with the activation of organized screening programs. **Figure 1**

Figure 1 – Colorectal cancer, males. *AIRTUM: estimate of incidence and mortality trends 2003-2018. Standardized rates new European population 2013*

There is also a significant reduction in incidence in the female sex (-1% / year in the period 2003-2018) [AIOM, 2018] **Figure 2**

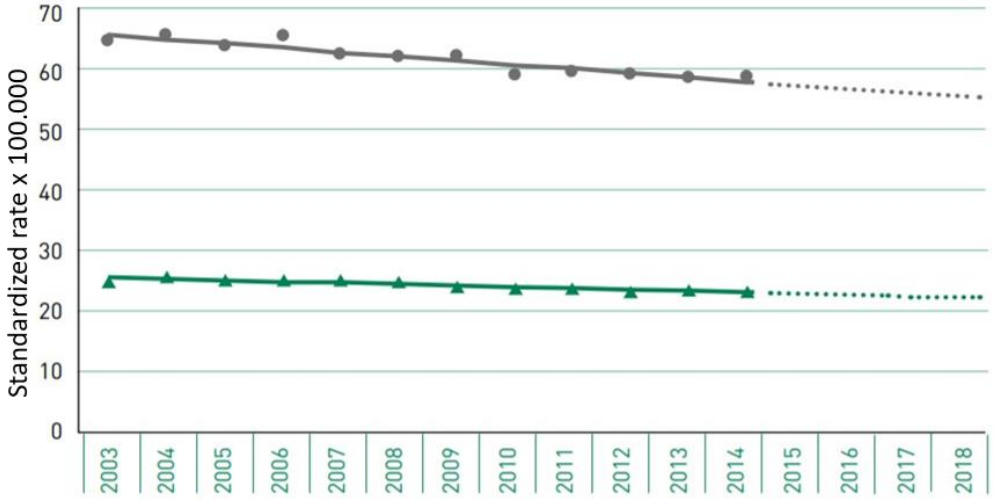


Figure 2 – Colorectal cancer, females. *AIRTUM: estimate of incidence and mortality trends 2003-2018. Standardized rates new European population 2013.*

Prevalence - There are over 471,000 patients with a previous diagnosis of colorectal cancer in Italy (52% male), ranking second among all tumors and 14% of all cancer patients. The 17% of these are actually more than 15 years after diagnosis (and 15% between 10 and 15 years), while the remaining quota is equally distributed within 2, between 2 and 5 and 5 and 10 years (about 23% for each period). Considering the stable and slight tendency to decrease of mortality observed at long term, it is above all the incidence (with the increasing tendency to the early diagnosis and the diffusion of screening programs in recent years) and the substantial good survival (tending to improvement) to condition the "numbers" of the people living with this type of diagnosis and their distribution throughout the clinical history, with obvious different needs for care and support in the follow-up. Most of these people are concentrated in the older age groups, with a proportion, over 75 years of age, of 2,914 cases per 100,000 inhabitants, double compared to the 60-74 age group and 8-10 times greater than the 45- 59.

Mortality In 2015, 18,935 deaths were observed due to colorectal carcinoma (ISTAT) (53% in men), neoplasm in second place in cancer mortality according to AIRTUM data (11% in males, 12% in females), and between the second and third place in the various ages of life. **Table 3** Mortality for this pathology is decreasing both among males (-0.7% /

year) and among females (-0.9%). Mortality also depends on the stage distribution at diagnosis, which is influenced by the availability of a population-screening program and by the level of care in each country. Since the 1980s, in several countries in Europe, North America and Asia, mortality has tended to decrease. This decrease might be attributable to the introduction of colonoscopy, which has improved detection and treatment of early lesions.

Rank	Male (age)			Females (age)		
	0-49	50-69	70+	0-49	50-69	70+
1°	Lungs (15%)	Lungs (29%)	Lungs (25%)	Breast (29%)	Breast (21%)	Breast (14%)
2°	Central Nervous System (11%)	Colorectal (10%)	Colorectal (11%)	Lungs (9%)	Lungs (15%)	Colorectal (13%)
3°	Colorectal (9%)	Liver (12%)	Prostate (10%)	Colorectal (7%)	Colorectal (10%)	Lungs (10%)
4°	Leukemia (7%)	Pancreas (7%)	Liver (7%)	Uterus (6%)	Pancreas (7%)	Pancreas (8%)
5°	Liver (7%)	Stomach (6%)	Stomach (7%)	Central Nervous System (6%)	Ovary (7%)	Stomach (6%)

Table 3 *– First five causes of cancer death and proportion of total cancer deaths by sex and age group. Pool AIRTUM 2010-2014*

- Survival Colorectal cancer has an overall favorable prognosis compared to many other solid tumors. The 5-year survival in Italy is 66% for the colon and 62% for the rectum, without any gender difference. It has high values among young patients, going from 69% between 15 and 44 years to 54% among the seniors (75+). The southern regions have survival rates that are approximately 6-8% lower than in the central-northern regions in males and 5-6% in females. Survival after 10 years from diagnosis is slightly lower than that at 5 years, with values of 64% for the colon and 58% for the rectum, homogeneous between men and women.

1.3 Risk Factors

About 80% of colorectal carcinomas arise from precancerous lesions (adenomas with a gradually increasing dysplastic component). Lifestyles and familiarity have long been called into question as factors in increasing the risk of incidence of these injuries. Among the first dietary factors stand out the consumption of red meats and sausages, refined flours and sugars, overweight and reduced physical activity, smoking and excess alcohol (Chan, et al., 2010). A protection, in addition to the control of the mentioned risk factors, is conferred by the consumption of fruits and vegetables, unrefined carbohydrates, vitamin D and calcium and by the administration of non-steroidal anti-inflammatory drugs at appropriate doses for a long time (Marshall, 2008). Further conditions of risk are Crohn's disease and ulcerative colitis (with an increased risk proportional to the extent of the disease) (HJ, 2008; Mellemkjaer L, 1995). About one third of colorectal cancers present characteristics of familiarity attributable to hereditary susceptibility: (Czene, et al., 2002) only a part of this familial risk (2-5%) is attributable to syndromes in which genetic mutations have been identified associated with increased risk of colorectal carcinoma (Leon, et al., 2006; Jasperson, et al., 2010). Among these the best known are:

- familial adenomatous polyposis (FAP) autosomal dominant transmission disease, due to the APC germline mutation, which involves the onset of hundreds of polyps around the age of 15-20 who tend to cancer over time (tumors of the skin appendages and osteomas are also associated).
- Lynch syndrome (Hereditary Non-Polyposis Colorectal Cancer, HNPCC), an autosomal dominant disease, due to mutations that reduce the effectiveness of the DNA repair system known as mismatch repair (the genes mainly involved are MLH1 and MSH2).

For individuals belonging to family groups at risk not jet framed in definite syndromes, the risk of developing colorectal cancer is about twice as high as in the general population if a first-degree blood relative is present, and more than triple if the latter has contracted the tumor before 50 years of age (Butterworth, et al., 2006)

1.4 Early Diagnosis

The colorectal screening program is applied at men and women aged 50 to 69 years and consists of an active prevention by means of a fecal occult blood test (and subsequent colonoscopy in positive cases) with regular control every 2 years. Screening can allow the detection and removal of precursors (adenomas) before transformation into carcinoma and the diagnosis of early-stage carcinomas, with a consequent reduction in mortality both due to reduction in incidence and to the detection of carcinomas in earlier stages, and therefore susceptible to healing after therapy. Thanks to screening programs, in 2015 about 3/4 of the Italian population were covered. In Italy there are marked differences between North and South / Islands.

1.5 Staging and Prognosis

There are numerous systems for the staging of colorectal cancer. In the past the most used was the one introduced by Dukes (Dukes, 1932) in 1932 and modified about 20 years later by Astler and Coller (Astler, et al., 1954). Currently, the TNM system is used and secondarily the AJCC (American Joint Commitments on Cancer) and UICC (Union International Contre le Cancer) classifications that exceed the limits of the previous classification, allowing to detect the primary tumor, lymph node involvement and distant metastases (Sobin, et al., 2010) **Table 4**.

T=primary tumour
TX–primary tumour cannot be assessed
T0–no evidence of primary tumour
Tis–carcinoma in situ: intraepithelial or invasion of lamina propria
T1–tumour invades submucosa
T2–tumour invades muscularis propria
T3–tumour invades through the muscularis propria into subserosa or into non-peritonealised pericolic or perirectal tissues
T4a–tumour penetrates the surface of the visceral peritoneum
T4b–tumour directly invades or is histologically adherent to other organs or structures

N=regional lymph nodes
NX–regional lymph nodes cannot be assessed
N0–no regional lymph node metastasis
N1a–metastasis in one regional lymph node
N1b–metastasis in two to three regional lymph nodes
N2a–metastasis in four to six regional lymph nodes
N2b–metastasis in seven or more regional lymph nodes

M=distant metastasis
MX–distant metastasis cannot be assessed
M0–no distant metastasis
M1a–distant metastasis to one site
M1b–distant metastasis to more than one site
Surveillance, Epidemiology and End Results Program data for 5-year stage-specific relative survival rates in colon cancers:[91,92]

- Stage I (T1, T2, N0): 97·1%
- Stage IIA (T3, N0): 87·5%
- Stage IIB (T4, N0): 71·5%
- Stage IIIA (T1, T2, N1): 87·7%
- Stage IIIB (T1, T2, N2): 75·0%
- Stage IIIB (T3, N1): 68·7%
- Stage IIIC (T3, N2): 47·3%
- Stage IIIC (T4, N1): 50·5%
- Stage IIIC (T4, N2): 27·1%

5-year stage-specific relative survival rates were similar for rectal cancer as compared with colon cancer.

Table 4 – TNM classification of colorectal cancer

The stage of the disease is the factor that correlates better than all with survival, which, at 5 years, is 94% for stage I, 82% for stage II, 67% for stage III, 11% for stage IV.

A limitation of the modern staging systems is the difficulty in identifying patients with a good prognosis from those with a poor prognosis (cancer, 2014). An example of this phenomenon is observed in the case of patients classified as stage II according to the TNM system. Although these are subjects without lymph node involvement, they do not always present a good prognosis after radical surgery. For a better risk stratification, it will be necessary to resort to prognostic factors related to tumor biology.

1.6 Therapies

Surgery is the most common treatment for colorectal cancer (AIOM, 2015). In patients with positive lymph nodes (stage III) after "curative" surgery, adjuvant chemotherapy containing oxaliplatin in combination with 5-fluorouracil / folinic acid (FOLFOX4 regimen) or capecitabine (XELOX regimen) determine a significant prolongation of survival compared to treatment with only 5-fluorouracil / folinic acid (Cutsem, et al., 2011) (Bokemeyer, et al., 2009). The introduction in the therapy of the new anti-angiogenic biological drugs (bevacizumab, aflibercept, ramucirumab) and anti-EGFR (cetuximab, panitumumab) resulted in a further significant improvement in the clinical efficacy of the treatments (Hurwitz, et al., 2004) (Loupakis, et al., 2014), with a survival that may exceed 30 months. The molecular characterization of patients with metastatic colorectal cancer based on the KRAS mutation test (codons 12 and 13 of exon 2) represented an important advance in the treatment of disease, allowing to exclude resistant patients from anti-EGFR monoclonal antibodies (mAb) therapy. Mutations of codons 12 and 13 of KRAS exon 2 represented until August 2013 the only criterion envisaged by the European and national regulatory agencies (EMA, AIFA) for the molecular selection of patients with metastatic colorectal cancer to be untreated with anti-EGFR drugs. Further investigations into molecular characterization allowed to identify other mutations that may be involved in resistance to treatment with anti-EGFR mAbs, represented by mutations in exons 3 and 4 of KRAS and in exons 2, 3 and 4 of NRAS. The determination of all these RAS mutations (exons 2, 3 and 4 of KRAS and NRAS) is currently required for the selection of patients for which is not recommended anti-EGFR mAb treatment. It was also shown that the presence of BRAF mutations, detectable in about 9% of patients with metastatic colorectal cancer, has a negative prognostic significance and is related to resistance to chemotherapy (Sorich, et al., 2015).

1.7 Molecular Alterations

Colorectal cancer (CRC) arises as the result of the accumulation of acquired genetic and epigenetic changes that transform normal glandular epithelial cells into invasive adenocarcinomas. A multisteps process transforms normal epithelium to benign neoplasms (adenomas and sessile serrated polyps), followed by invasive carcinoma, and eventually metastatic cancer. (Fearon, et al., 1990) (Grady, 2005) **Figure 4**

This process may take years to decades to escape the multiple regulatory layers of the cells and to fully develop (Kinzler, et al., 1996).

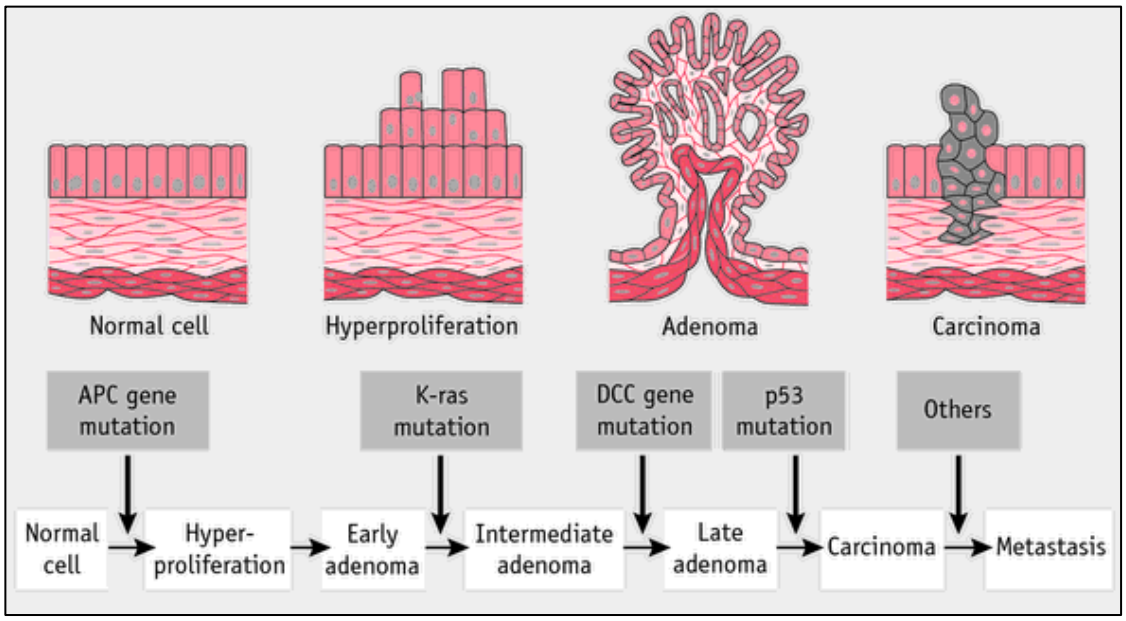


Figure 4 – Adenoma carcinoma sequence. *The development of carcinoma from adenomatous lesions is referred to as the adenoma-carcinoma sequence.*

The average rate of genomic mutation in normal human cells is estimated to be ~2.5x10-8 mutations/nucleotide/generation (Nachman, et al., 2000) (Roach, et al., 2010). However, this rate is higher in cancer cells due to the sequential accumulation of multiple mutations during cell divisions forming a so-called 'mutator phenotype' (Loeb, et al., 2003). Accordingly, mutations in genes that regulate cell cycle checkpoints, and/or cellular responses may increase mutation rates to the level commonly observed in human tumors

(Loeb, et al., 2003). The 'mutator phenotype' may have various manifestations, including point mutations, CIN (Chromosomal Instability), MSI (Microsatellite Instability), CIMP (CpG Island Methylator Phenotype pathways) (Loeb, et al., 2003).
Specifically, the main alterations of the signaling pathways in colorectal carcinoma cells are found for the following genes:

- APC is first gene commonly mutated in familial/inherited and sporadic colon cancer, associated with Familial Adenomatous Polyposis (C. Soravia, 1988). The APC tumour suppressor gene normally blocks transition from G1 to S phase of the cell cycle. The Wnt signaling pathway maintains native stem cells in their undifferentiated state at the base of the colonic crypts, which contributes not only to the survival of normal stem cells but also to the survival of cancer cell stem cells. β-Catenin is a major player in the Wnt signaling pathway. Unmutated APC induces degradation of β-catenin and therefore functions as a negative regulator of the Wnt signaling pathway. (Polakis, 1997). In most colorectal carcinomas, in fact, the APC protein is inactivated by mutations and, consequently, incapable of regulating β-catenin. β-catenin in the nucleus forms complexes with transcription factors, acting as a co-activator of genes involved in cell growth and proliferation, as well as extracellular proteases, used in invasion and metastasis processes. (He, et al., 1998) (Tetsu, et al., 1999).

- *TP53* is one of the most important tumour suppressor genes and is the main cell cycle checkpoint regulator (Pino, et al., 2010). Loss of 17q, where *TP53* is located, is a frequent event in CRCs because it plays a critical role in the canonical adenoma–adenocarcinoma sequence. P53 inactivation drives tumor progression, allowing increased cell proliferation. Indeed, the transition from adenoma to invasive carcinoma is usually associated with *TP53* inactivation.

- 18q Loss of Heterozygosity (LOH) is the most frequent cytogenetic alteration in colorectal cancer and is observed in up to 70% of CRCs, and it may influence CRC

behaviour by deregulating TGF-ß signaling (Popat, et al., 2005). LOH is defined as loss of one of the two copies or alleles of a gene. Often the remaining allele is affected by a mutation. Two tumour suppressor genes affected by this cytogenetic alteration are *DCC* and *SMAD4.* The DCC (Deleted in Colorectal Carcinoma) gene is located on the long arm of chromosome 18 (18q21.3) and encodes for the transmembrane protein DCC. DCC is a "conditional tumour suppressor gene." Approximately 70% of CRCs show LOH in the DCC gene region. Netrin-1 is produced deep in the crypts of the colorectal mucosa. When epithelial cells differentiate and move toward the surface, the concentration of netrin-1 decreases. This concentration gradient is felt to contribute to the normal process of apoptosis and sloughing of epithelial cells. When the DCC gene is mutated, netrin-1 will not bind to DCC transmembrane protein, resulting in abnormal cell survival (Mehlen, et al., 2004). SMAD are a series of proteins that normally work by forming a trimer composed of two SMADs regulated by the receptors and a co-SMAD (Massagué, et al., 2005). This complex acts as a transcription factor with a predominantly tumor suppressor mechanism. It was observed that in half of colorectal carcinoma without mutations of the mismatch repair system, TGF-β signaling is abolished by inactivation of SMAD4 or its partner factors SMAD2 and SMAD3 (Leary, et al., 2008). However, this is a late mutation, which is observed in conjunction with the transition from large adenoma to high-grade dysplasia /invasive carcinoma (Grady, et al., 1998)

- *KRAS*, a member of the *RAS* family of proto-oncogenes, is the most frequently mutated gene in all of human cancer and an important oncogene in CRC. The KRAS protein is a downstream effector of EGFR that, through BRAF, activates the mitogen activated kinase (MAPK) pathway and promote cell growth and survival. Mutations in *KRAS* codons 12 or 13 occur in approximately 40% of colorectal cancers and lead to constitutive signaling by impairing the ability of GTPase activating proteins to hydrolyze KRAS-bound GTP (Downward, 2003). *KRAS* mutations occur after *APC* mutations in the adenoma-to-carcinoma progression sequence, but are still a relatively early event in tumorigenesis. The *BRAF* gene, mutated in ~10-15% of

colorectal cancers, encodes a protein kinase that is the direct downstream effector of KRAS in the Ras/Raf/MAPK signaling pathway. Most of *BRAF* mutations are a single base change resulting in the substitution of glutamic acid for valine at codon 600 (Siena, et al., 2009). Furthermore, RAS can also interact with other signaling pathways. For example, is known its ability to activate PI3K and to stimulate the expression of the glucose channel GLUT1 (Yun, et al.) is known, in fact making this proto-oncogene able to influence not only cell growth, but also metabolism, forcing the cell towards anaerobic energy production (Warburg effect).

- *PI3K* Mutations in phosphatidylinositol 3-kinase pathway genes are observed in up to 40% of colorectal cancer and are nearly mutually exclusive of one another (Parsons, et al., 2005) The most frequent mutations of the PI3K pathway occur in the p110α catalytic subunit *PIK3CA*, which are detected in up to 32% of colorectal cancers and may promote the transition from adenoma to carcinoma. Mutations are also observed in *PTEN*, a tumor suppressor gene that negatively regulates PI3K signaling; loss of PTEN expression occurs in 30-40% of CRCs (Yu, et al., 2014).

2 INTRODUCTION: ROLE OF TRAP1 IN COLORECTAL CANCER

2.1 Physiological Role of TRAP1

TRAP1 (TNF Receptor Associated Protein 1) is a mitochondrial Heat-Shock Protein, initially identified as a factor associated with the TNF receptor, from which it took its name (Matassa, et al., 2012). From gene sequencing analysis it was found to be identical to HSP75, a protein of the HSP90 family, whose functions range from refolding of denatured proteins to signal transduction, and in some cases in protein degradation processes (De Roock, et al., 2010).

The gene encoding TRAP1 is located on chromosome 16 and contains 18 exons. The final protein is composed of 704 amino acids and has a weight of 75 KDa and contains three domains:

- N-terminal domain, called MTS, responsible for mitochondrial protein localization.
- Intermediate domain, which contains 4 binding sites for ATPases.
- C-terminal domain similar to that of the HSP90

Several studies have shown that TRAP1 is expressed both in transformed cells and in different cell lines of normal tissues. It is also highly homologous to several members of the HSP90 family (26% identical and 45% homologous with respect to HSP90) (Song, et al., 1995) . HSP90 is a ubiquitous chaperone, highly conserved in evolution (Gupta, 1995; Lindquist, et al., 1988), involved in numerous cellular processes (Pearl, et al., 2006) : many molecules involved in different signaling processes need HSP90 to stay in an active state. Like all proteins in the HSP90 family, TRAP1 is also a molecular chaperonin. This class includes all the proteins able to bind the unstable conformers of other proteins, called clients, and facilitate their correct function by folding, oligomeric assembly, transport to

specific compartments. They are also capable of inducing degradation under certain conditions, including the impossibility of achieving correct folding (Langer, et al., 2003; Lindquist, et al., 1988). All these functions are performed at the expense of the energy of the cell in the form of ATP. In fact, all chaperonins have ATP-asica activity. Immunofluorescence studies show that TRAP1 is localized in mitochondria (Felts, et al., 2000). Here it interacts with HSP90 to antagonize the action of cyclophilin D, a proapoptotic molecule, favoring cell survival (Calderwood, et al., 2005;) The increase in apoptosis resistance that TRAP1 is able to induce is consistent with its increase in expression in numerous tumors and with its reduced expression in normal tissues (Kang, et al., 2007). However, TRAP1 was also found outside the mitochondria. A particularly interesting localization is at the level of proteasomes present at the interface with the endoplasmic reticulum, where it interacts with TBP7, a regulatory subunit of the complex, taking part in the quality control of mitochondrial proteins, with regulation of their degradation (Amoroso, et al., 2012). TRAP1 is also found in the endoplasmic reticulum, associated with ribosomes, where it is thought to be capable of performing regulatory functions:

- Reduction of cap-dependent protein synthesis through kinase-dependent pathways (such as GCN-2 and PERK) that phosphorylate eIF2 (Bazzaro, et al., 2006) .
- Increase in IRES-dependent protein synthesis, probably to facilitate the expression of stress response genes.
- Protection against stressful events by induction of Unfolded Protein Response (Maddalena, et al., 2013; Siegelin, et al., 2011).

The cytoprotective action of TRAP1 is also expressed through a series of changes in cellular metabolism that lead to a reduced production of ROS and consequently a reduction in the phenomena of damage from radicals (Montesano Gesualdi, et al., 2007; Im, et al., 2007).

2.2 TRAP1 in Colorectal Cancer

The prognostic role of TRAP1 in colorectal cancer has been studied in recent years. Recent data suggest how TRAP1 may play an important role not only in tumorigenesis, but also in chemoresistance. Initial evidence has shown that TRAP1 is upregulated in about 50-60% of colorectal carcinomas (Han, et al., 2014). In a 2012 study it was shown that the increase in TRAP1 expression levels was significantly correlated with an increase in the number of lymph node metastases, at an advanced stage and reduced overall survival (Gao, et al., 2012). This suggests that TRAP1 plays an important role in the progression of colorectal cancer, suggesting TRAP1 as a predictive biomarker of poor prognosis. In this context, a study carried out in the department of internal medicine of Seoul - Korea, showed a relationship between the overexpression of TRAP1 and resistance to chemotherapy. A recent study also showed that overall survival in colorectal carcinoma is significantly reduced when TRAP1 and its network of client proteins are overexpressed (Maddalena, et al., 2017). This study analyzed the expression of TRAP1 and its proteome in colorectal cancer demonstrating that:

- TRAP1 upregulation occurs at the level of the transition between low and high grade polyps;
- TRAP1 is co-expressed with most of its client proteins in about 60% of colorectal carcinomas;
- The upregulation of TRAP1 and its network of client proteins is associated with a poor prognosis;
- From a clinical point of view it is possible to identify a signature of 7 proteins (TRAP1, IF2α, eF1A, TBP7, MAD2, CDK1 and β-Catenine) which allows to select patients with metastatic colon carcinoma with an unfavorable prognosis.

2.3 TRAP1 Client Protein Network

TRAP1 is responsible for several functions in cancer cells; indeed this chaperonin is important in stress, apoptosis, drug resistance (Maddalena, et al., 2013; Costantino, et al., 2009), protein homeostasis, stem cell maintenance (Lettini, et al., 2016) and, finally, cell cycle regulation (Sisinni, et al., 2017). All these functions can be performed through the quality control of specific TRAP1 client proteins and consequently through the modulation of their expression / ubiquitination. The network of TRAP1client proteins has several members, many of which are involved in cell cycle regulation, apoptosis, protein homeostasis, stem cell maintenance and metabolism control. In particular:

SORCIN- A calcium-binding protein that regulates the activity of type L voltage-dependent calcium channels. TRAP1 plays a quality control role only on the mitochondrial isoform (18 KDa, a member of the EF-hand family), which plays a role in chemoresistance and evasion of apoptosis, probably through the regulation of calcium homeostasis (Landriscina, et al., 2010).

BRAF - Oncogene involved in growth, invasiveness and development of metastases (Karasarides, et al., 2004) .TRAP1 regulates the synthesis, ubiquitination and degradation of BRAF (Condelli, et al., 2014). The evidences supporting this role are the following:

- BRAF levels are extremely reduced in cells with silenced TRAP1, while its mRNA is normally present.
- High levels of TRAP1 favor the synthesis of BRAF.
- The silencing of TRAP1 leads to an increase of BRAF ubiquitination.
- BRAF levels are increased by the expression of a mutated form of TRAP1 whose function is limited to the endoplasmic reticulum.

TBP7 - It is an ATPase related to the S6 subunit of the proteasome. Interacts with TRAP1 at the proteasomal level. It seems, however, that this interaction also exists at the

endoplasmic level. The hypothesis is that this interaction ensure that unfolded proteins are directed to the proteosomal destruction (Tsai, et al., 2010).

β-Catenin - important in the Wnt / Catenin signaling system, this protein acts as a cell-cell interaction molecule, and is degraded by APC when it detaches from the membrane. This degradation is inhibited when Wnt signaling molecule binds to its Frizzled receptor, consequently APC is inhibited and β-Catenin is able to translocate in the nucleus for the pathway activation (Lettini, et al., 2016). Recent studies suggest that TRAP1 prevents the phosphorylation and degradation of β-Catenine, shifting the balance of its pathway towards activation (Maddalena, et al., 2017).

CDK - Cyclin-dependent kinases play a key role in cell proliferation regulation. CDK1, interacting with cyclin B, forms a complex known as Mitosis Promoting Factor (MPF). This complex is upregulated in the S and G2 phases of the cell cycle, and then significantly reduced at the end of phase M. Recent work has shown that CDK1 is a client protein of TRAP1, which promotes cell proliferation by performing a quality control on the cyclin-dependent kinase (Sisinni, et al., 2017).

MAD2 - It is part of the checkpoint proteins of the mitotic spindle, functioning as an ancillary tubulin protein. It acts by delaying the anaphase until the pairs of sister chromatids are aligned. Indeed, it represents a protection mechanism for the non-disjunction of the chromatids. Recent observations have shown that MAD2 is a client protein of TRAP1 (Sisinni, et al., 2017).

IF2 - It is a protein synthesis initiation factor which mediates the binding of tRNA to the ribosome in a GTP-dependent manner. It is crucial in the formation of the 43s complex that precedes the onset of protein synthesis. Its function is regulated by TRAP1 (Maddalena, et al., 2017).

P70 / S6 Kinase - is a serine / threonine kinase, an enzyme that acts downstream of PI3K, phosphorylating the S6 subunit of the ribosome and inducing protein synthesis. Together with AKT, it is part of the PI3K / AKT / p70S6K signaling pathway. Recently it has been shown that the silencing of TRAP1 promotes cellular motility in vitro, but it compromises the ability of cells to resist stress conditions and that these effects are mediated by the AKT / p70S6K pathway (Agliarulo, et al., 2015). The study has indeed observed that:

- Inhibition of AKT / p70S6K activity reduces migration of TRAP1 silenced cells.
- TRAP1 regulates the expression of both AKT and p70S6K at the post-transcriptional level.
- Nutrient deprivation affects the activity of p70S6K compromising cell migration only in cells with TRAP1 deficiency.
- TRAP1 silencing modulates the expression of genes involved in tumor cell migration and in epithelial-mesenchymal transition.

In particular, a correlation was observed between TRAP1 and AKT in colorectal cancer. This study opened new perspectives on the role of TRAP1 in cell migration, tumor progression and metastatic mechanism.

2.4 Role of TRAP1 in the Metabolic Rewiring

Recent works have demonstrated a probable role of TRAP1 in the regulation of cellular metabolism, with conflicting results; also, these effects appear to be mediated mainly by its mitochondrial form. Independent studies have shown that TRAP1 has the ability to reduce oxidative phosphorylation. In particular, a first study shows that this phenomenon seems to be due to the interaction between TRAP1 and the c-Src kinase mitochondrial fraction, a regulatory enzyme that constantly stimulates the activity of the respiratory complex IV (Miyazaki, et al., 2003; Ogura, et al., 2012). It was shown that the two proteins colocalize in the mitochondria and that they are able to regulate each other negatively. Specifically, the phosphorylated (active) form of mitochondrial c-Src is greatly reduced if TRAP1 is overexpressed, whereas in TRAP1 KO cells the levels of active c-Src increase markedly. According to the authors, it is reasonable to hypothesize that the interaction

between TRAP1 and c-Src blocks the enzymatic activity of the kinase, while the phosphorylation of TRAP1 by c-Src breaks the interaction between these two proteins. It is interesting to note that there appears to be no interactions between TRAP1 and the cytosolic form of c-Src (Yoshida, et al., 2013). Further data from the same paper show that the regulatory action of TRAP1 on mitochondrial metabolism is impossible in the absence of c-Src. TRAP1 plays a role of tumor suppressor because its expression is downregulated in specific tumors, including ovarian, renal and cervical carcinoma, in parallel with a condition of oxidative respiration. In the second study the peculiar interaction between TRAP1 and the respiratory complex II was demonstrated (Sciacovelli, et al., 2013; Rasola, et al., 2014).Indeed , chaperonin inhibits the enzymatic activity of succinate: ubiquinone reductase, and this inhibition involves a partial reduction of oxidative metabolism. However, starting from the evidence that TRAP1 is upregulated in numerous neoplasms, the authors show its opposite role in carcinogenesis. In fact, the inhibition of the respiratory chain complex II causes increased availability of succinate which acts as an oncometabolite, blocking the prolyl-hydroxylase which under normal conditions induces the degradation of HIF-1α (Yang, et al., 2013) . Therefore, TRAP1 would perform an oncogenic action as its upregulation causes inhibition of succinic dehydrogenase and respiratory activity, succinate accumulation, HIF-1α activation, and tumorigenesis induction. This different interpretation of the oncogenic or oncosuppressive role of TRAP1 is currently the subject of further studies and could be interpreted on the basis of recent data showing that the role of TRAP1 in tumorigenesis is context-dependent. Some neoplasms, with a prevalent glycolytic metabolism, activate the expression of TRAP1 using its oncogenic role, while other tumors with a prevalent oxidative metabolism downregulate its expression using it as an oncosuppressor. Two further studies support this interpretation. One has demonstrated the ability of TRAP-1 to act as an inducer of aerobic glycolysis in tumor cells where its expression levels are high (Chae, et al., 2012). In fact, TRAP1 downregulation by siRNA reduces the ATP and lactate production in the tumor lines and this occurs through the recruitment of hexokinase II in the mitochondria tumor cells. So, TRAP1 would be able to control pathways for the perception of the cellular energetic state by AMPK kinase dephosphorylation. The second

study analyzed the expression of TRAP1 in ovarian cancer demonstrating that the levels of chaperonin are reduced in parallel with the increase in grading and stage, thus assuming an oncosuppressive role. The downregulation of TRAP1 is associated with the increase in respiratory activity and with resistence to platinum. This study therefore demonstrates that the downregulation of TRAP-1 can represent a molecular mechanism that the ovarian carcinoma cell uses for the progression towards a pharmaco-dependent phenotype and that this process is due to a metabolic remodeling with acquisition of a predominantly oxidative metabolism (Matassa, et al., 2016) .

All these evidences, although not yet conclusive, therefore suggest that the role of TRAP1 in carcinogenesis is context-dependent and that the tumor cell can modulate TRAP1 expression related to its metabolic needs and extracellular environment. Moreover, additional evidence supports the role of TRAP1 in favoring glycolytic metabolism Indeed, many human malignant tumors (i.e., colorectal, breast, nasopharyngeal and pulmonary carcinomas), with high TRAP1 expression, are characterized by a predominantly glycolytic metabolic profile, while other tumors (i.e., ovarian, renal and cervical carcinomas) with low TRAP1 expression levels by an oxidative metabolism. **Figure 5**

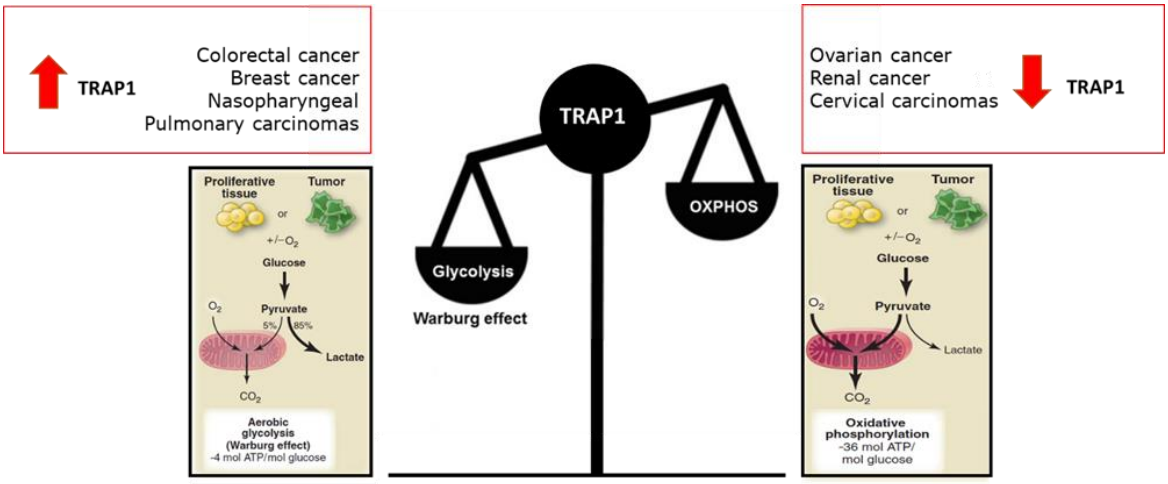


Figure 5 – TRAP1 acts as a metabolic balance.

3 INTRODUCTION: METABOLISM OF THE TUMOR CELLS

3.1 Normal Cellular Metabolism

Metabolism is the set of life-sustaining chemical reactions in organisms. The three main purposes of metabolism are: the conversion of food to energy to run cellular processes; the conversion of food/fuel to building blocks for proteins, lipids, nucleic acids, and some carbohydrates; and the elimination of nitrogenous wastes. The chemical reactions of metabolism are organized into metabolic pathways, in which one chemical is transformed into another by a sequence of enzymes. Enzymes are crucial in metabolism and allow the fine regulation of metabolic pathways to maintain a constant set of conditions in response to changes in the cell's environment, a process known as homeostasis.

Metabolic reactions may be categorized as anabolic and catabolic:

- Anabolism is a constructive metabolic process in which cells use energy to construct molecules such as enzymes and nucleic acids and perform other essential life functions. Anabolism involves three basic stages: firstly, the production of precursors such as amino acids, monosaccharides, isoprenoids and nucleotides; secondly, their activation into reactive forms; and thirdly, the assembly of these precursors into complex molecules.

- Catabolism is the metabolic process by which the cell breaks down complex molecules. The purpose of catabolic reactions is to provide the energy and components needed by anabolic reactions.

Usually, catabolism releases energy, and anabolism consumes energy. In normal cells, ATP is the main energy sourse generated through catabolism of carbohydrates, proteins, and fats. Carbohydrates are broken down to glucose that is converted to pyruvate and

acetyl-coenzyme A (CoA). Acetyl-CoA is metabolized to a series of intermediates in the tricarboxylic acid (TCA) pathway, or "Krebs cycle," releasing ATP and high-energy carriers (nicotinamide adenine dinucleotide phosphate [NADPH]) that result in additional ATPs. Energy is released in the form of ATPs during aerobic glucose oxidation to carbon dioxide and water. Protein is broken down to amino acids that help build pyruvate and acetyl-CoA molecules. Fats are catabolized to a small-chain three-carbon molecule, glycerol, and fatty acids that help build pyruvate and acetyl-CoA molecules. Both anabolism and catabolism pathways are regulated by several hormones (glucagon, glucocorticoids, insulin, and growth hormone) (Deberardinis, et al., 2008) . Fora therapeutic perspective, the need to understand cancer cell metabolism is critically important. Therapeutic strategies for cancer treatment involve targeting several metabolic pathways, including glycolysis, the Krebs cycle, oxidative phosphorylation (OXPHOS), glutamine metabolism, fatty acid oxidation, nucleic acid synthesis, lipid synthesis, and amino acid metabolism.

3.2 Metabolic Rewiring in Cancer

Energy metabolism reprogramming, has been considered as an emerging hallmark of cancer (Hanahan, et al., 2011). Tumor metabolic reprogramming involves the enhancement of key metabolic pathways such as glycolysis, pentose phosphate pathway, glutaminolysis and lipid, nucleic acid and amino acid metabolism (Ward, et al., 2012) **Figure 6**. It is well established that normal cells get energy first via t glycolysis in the cytosol that is followed by mitochondrial oxidative phosphorylation under aerobic conditions. When oxygen availability is reduced, the cells rely on glycolysis rather than oxygen-consuming mitochondrial metabolism for energy supply. However, cancer cells prefer to perform glycolysis in the cytosol even in the presence of oxygen, a phenomenon first observed by Otto Warburg (Warburg, 1956; Warburg, 1956) and now famously known as ''Warburg effect'' or ''aerobic glycolysis''. As a result, many transformed cells derive a substantial amount of their energy from aerobic glycolysis, converting most incoming glucose to lactate rather than metabolizing it in the mitochondria through oxidative phosphorylation (Semenza, et al., 2011). Although ATP production by glycolysis can be

more rapid than oxidative phosphorylation, it is less efficient in terms of ATP generated per unit of glucose consumed (~18-fold). This paradoxical phenomenon, has been observed across several tumor types and is one of the first recognized biochemical hallmarks of cancer cells. Usually, cancer cells are highly glycolytic and they avidly take up more glucose than do normal cells from outside. The increased uptake of glucose is facilitated by the overexpression of several isoforms of membrane glucose transporters (GLUTs). Although the Warburg effect was discovered decades ago, use of the glycolytic pathway as a potential target for anticancer drug development was not recognized by the scientific community. However, the Warburg effect was used in cancer diagnoses and in evaluating the drug response in cancer treatment. Experimental demonstration of the higher uptake of glucose in tumors and metastasis came from positron emission tomography (PET) imaging, one of the most commonly used imaging techniques used for diagnosing tumor growth and response to therapy (Groves, et al., 2007). Initially, it was believed that the Warburg effect resulted from defects in the mitochondria1 function of cancer cells. However, this effect is also exhibited by tumor cells with intact and functional mitochondria, suggesting that their preference for glycolysis might confer benefits on them. The hypotheses put forward to explain this effect are:

- In conditions of aerobic glycolysis, cells can live in conditions of fluctuating oxygen tension (due to inconstant hemodynamics of distant blood vessels) that would be lethal for cells that rely on oxidative phosphorylation (OXPHOS) to generate ATP (Pouyssegur, et al., 2006)
- Cancer cells generate lactate from pyruvate fermentation; lactate acidifies the tumor environment (Koukourakis, et al., 2006), promoting tumor invasion (Swietach, et al., 2007) , and suppressing anticancer immune effectors (Fischer, et al., 2007)
- Tumors can metabolize glucose through the pentose phosphate pathway (PPP) to generate nicotinamide adenine dinucleotide phosphate (NADPH) that ensures cellular antioxidant defenses against a hostile microenvironment and chemotherapeutic agents (Gatenby, et al., 2004).

- Cancer cells use intermediates of the glycolytic pathway for anabolic reactions (for instance, glucose 6-phosphate for glycogen and ribose 5-phosphate synthesis, dihydroxyacetone phosphate for triacylglyceride and phospholipid synthesis, and pyruvate for alanine and malate synthesis) (Gatenby, et al., 2004).

From these observations it is clear that, although in the past the metabolism of tumor cells has been identified in anaerobic glycolysis, the metabolic reprogramming of the tumor cell is much more complex and articulated.

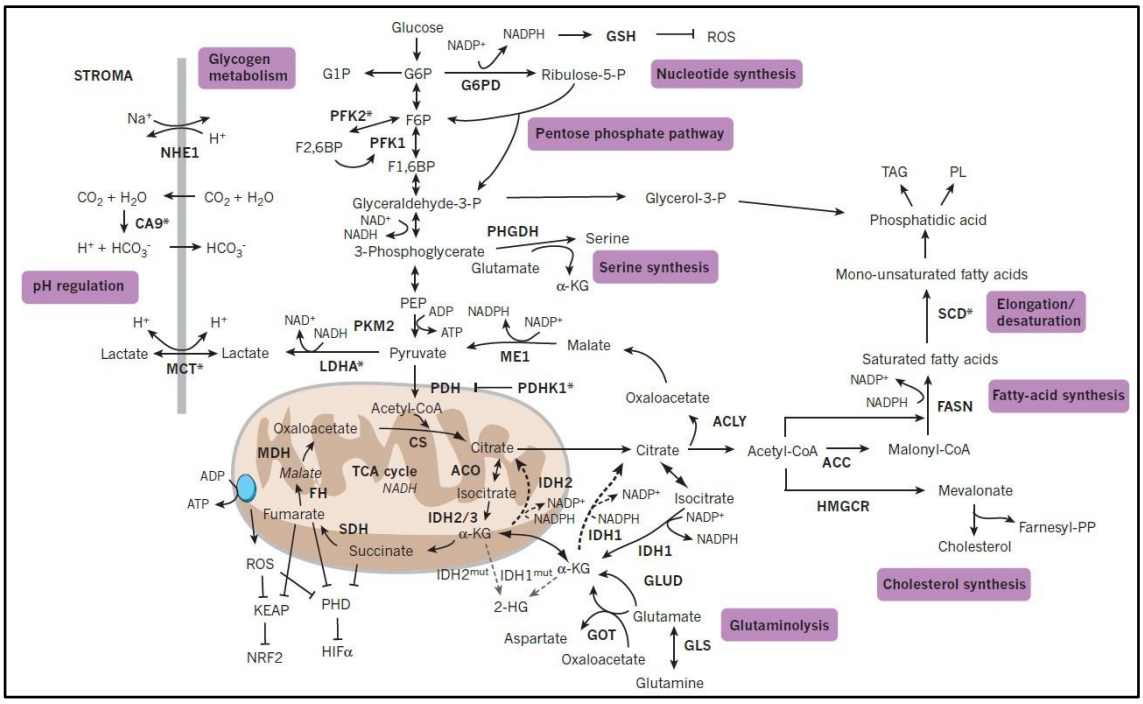


Figure 6 – Overview of metabolic activities in cancer cells. *Main reactions that cancer cells use to produce energy and intermediates for the synthesis of biomolecules.*

3.3 Main Signaling Pathways Involved into Metabolic Rewiring

Numerous studies have been conducted to try to understand what are the control points of metabolic rewiring in the cancer cell. Currently, signaling pathways that seem to have a crucial role are:

PI3K / Akt / mTORC1 - This pathway is classically known for its ability to inhibit apoptosis. It is antagonized by PTEN. The effects of its activation greatly affect the metabolism:

- Akt promotes the entry of glucose into the cell, inducing GLUT4 exposure (Kohn, et al., 1996).
- Akt is able to increase the activity of two glycolysis control enzymes. Through phosphorylation it enhances the activity of hexokinase (which causes glucose to remain within the cell) and phosphofructokinase 2, which activates phosphofructokinase 1 (Deprez, et al., 1997; Gottlob, et al., 2011).
- Akt also has a role in cellular anabolism. The cancer cell needs not only to produce energy, but also to synthesize new molecules for growth. The intermediate most used in synthesis reactions (especially lipids) is acetyl-CoA, which is formed only in the mitochondria. To transport it into the cytoplasm it must be combined with oxalacetate to form citrate. The citrate is then brought outside of the mitochondria and here it is splited to release the acetyl-CoA. This last reaction is catalyzed by ATP-citrate lyase, whose action is promoted by Akt (Bauer, et al., 2005; Berwick, et al., 2002; Hatzivassiliou, et al., 2005).
- mTORC1 is a powerful stimulator of mitochondrial biogenesis, especially of the synthesis of amino acids. Specifically, it activates PGC-1α which stimulates the transamination of oxalacetate to alpha-ketoglutarate. This intermediate can be converted into aspartate, which can be used to synthesize asparagine, glutamine and proline (Cunningham, et al., 2007; Ramanathan, et al., 2009).
- mTORC1, finally, activates the transcription factor SREBP, whose effect is the induction of lipid biosynthesis (Düvel, et al., 2010).

Myc – It is known to enhance glycolysis through the activation of glycolytic genes (such as *HK2*, *GAPDH*, *ENO1* and *PK*, among others) and glucose transporters (*SLC2A1*, *SLC2A2* and *SLC2A4*) (Dang, et al., 2006; Osthus, et al., 2000) In addition, MYC promotes lactate production and export, increasing the gene expression of *LDHA* and lactate transporter *MCT1* (Adhikary, et al., 2005; Rimpi, et al., 2007;). On the other hand, transformed cells exhibit increased MYC-dependent glutaminolysis and glutamine dependency (Wise, et al., 2008; Gao, et al., 2009). Indeed, MYC has been described as the main oncoprotein responsible for inducing a transcriptional program that promotes glutaminolysis and triggers cellular addiction to glutamine as a bioenergetic substrate.
This glutamine addiction leads tumor cells to reprogram intermediate metabolism for the maintenance of mitochondrial tricarboxylic acid (TCA) cycle integrity (Wise, et al., 2008). Moreover, high levels of MYC promote mitochondrial biogenesis and function, both increasing the rate of oxygen consumption and the energy production required for rapid cell proliferation (Wahlstrom, et al., 2015; Li, et al., 2005;). High glutaminolysis rate results in the robust production of NADPH, which is needed to fulfill the requirements for cell proliferation (DeBerardinis, et al., 2007;)

RAS - It is one of the most important drivers in colorectal cancer. Its metabolic action is remarkable: it has been shown that reducing glucose concentrations in culture medium of colorectal carcinoma cells favors the onset or selection of clones with RAS mutations, which allow cells to survive by increasing expression of GLUT1 (Yun, et al., 2009). It was also been observed that the increase in glycolytic metabolism induced by RAS does not arise as a consequence of a defect in mitochondrial oxidative metabolism, which indeed seems to be preserved (Guo, et al., 2011; Weinberg, et al., 2010).

p53 -Although the transcription factor and tumour suppressor p53 is best known for its functions in the DNA damage response (DDr) and apoptosis, it is becoming clear that p53 is also an important regulator of metabolism (Vousden, et al., 2009). p53 activates the expression of hexokinase 2 (HK2), which converts glucose to glucose-6-phosphate (G6P)

(Mathupala, et al., 1997). G6P then either enters glycolysis to produce ATP, or enters the pentose phosphate pathway (PPP), which supports macromolecular biosynthesis by producing reducing potential in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and/or ribose, the building blocks for nucleotide synthesis. However, p53 inhibits the glycolytic pathway by upregulating the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), an enzyme that decreases the levels of the glycolytic activator fructose-2,6- bisphosphate (Bensaad, et al., 2006). Wild-type p53 also supports the expression of PTEN, which inhibits the PI3K pathway, thereby suppressing glycolysis (Stambolic, et al., 2001). Furthermore, p53 promotes oxidative phosphorylation by activating the expression of sCO2, which is required for the assembly of the cytochrome *c* oxidase complex of the electron transport chain. Thus, the loss of p53 might also be a major force behind the acquisition of the glycolytic phenotype.

It is therefore clear that the same signaling pathways, whose alteration was believed to involve only an increase in cell growth, are also responsible for the change in the metabolism of the tumor cell. These data refuse the theory that metabolic alterations are always secondary to damage of traditional energy-producing systems suggesting a radical change in the concept that metabolism is independent from cell replication control.

3.4 Mitochondrial Metabolism

Mitochondrial function is essential for cancer cells since it is involved in numerous crucial cellular processes such as ATP generation, regulation of programmed cell death, and regulation of signal transduction pathways through ROS production, modulation of cytosolic calcium levels and trafficking of small metabolites. The mitochondrial respiratory chain, located in the mitochondrial inner membrane, comprises four complexes (I to IV) that are responsible for the oxidation of the reducing equivalents in the form of NADH or FADH2 and the reduction of molecular oxygen (final electron acceptor) to water. Among the metabolic pathways that take place in the mitochondria, the tricarboxylic acid (TCA) cycle is a step of pivotal importance for the entire cellular metabolism and, in particular, for oxidative metabolism. Remarkably, TCA cycle provides

precursors for the biosynthesis of lipids, nucleic acids and proteins, as well as reducing equivalents (NADH and FADH2) for ATP production. Mutations in several genes coding for enzymes of the TCA cycle including isocitrate dehydrogenase (Ward, et al., 2010; Kang, et al., 2009), succinate dehydrogenase and fumarate hydratase (Xiao, et al., 2012) are associated with some tumor types, leading to the dysfunction of the TCA cycle and the accumulation of its substrates (Gaude, et al., 2014; Desideri, et al., 2015; Chen, et al., 2012). Interestingly, it has been shown that increased levels of TCA cycle intermediates fumarate and succinate can affect α-ketoglutarate-dependent histone and DNA demethylases, HIF stabilization, and cellular responses to O2 depletion (Selak MA, et al., 2005).

3.5 Reverse Warburg Effect: Environmental Implication on Metabolic Rewiring

Altered tumor metabolism is not simply the final outcome of some combination of cellular genetic alterations. Instead, a non-genetic component in the form of the tumor microenvironment must additionally be considered as a component in the equation that influences metabolic changes in cancer cells (Marusyk, et al., 2012). The "reverse Warburg effect" is a new term for "parasitic" cancer metabolism. (Xie, et al., 2015; Pavlides, et al., 2009). The glycolytic product, lactic acid, secreted by cancer cells or fibroblasts is also used by neighboring cancer cells to make citric acid and sustain cancer progression. It was proposed that cancer cells act as metabolic parasites because they obtain nutrients from host cells by inducing catabolic processes. The tumor stroma or the tumor microenvironment, composed of fibroblasts, adipocytes, endothelial cells, and macrophages, becomes the source of fuel for tumor growth. Tumors use energy-rich metabolites from the microenvironment. Tumor cells constantly interact with their microenvironment (Hui, et al., 2015). In addition to glycolysis, cancer cells will use fatty acids from adipocyte tissues for energy. Other stromal-derived metabolites that promote oxidative mitochondrial metabolism and ATP production in epithelial cancer cells are glutamine and ketones. Monocarboxylate transporters (MCTs) shuttle L-lactate between cancer-associated stromal cells and cancer cells (Sanita, et al., 2014). MCT4, is

responsible for exporting L-lactate out of stromal cells, and MCT1, localized in epithelial cancer cells, is responsible for L-lactate uptake. **Figure 7**

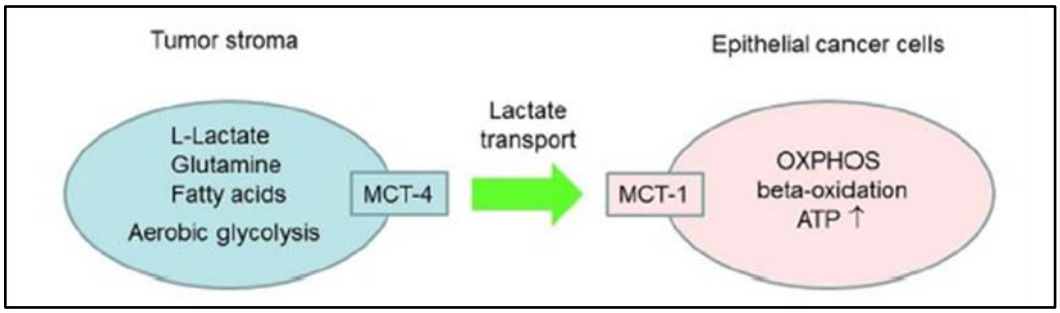


Figure 7 – The reverse Warburg effect in cancer metabolism

4 INTRODUCTION: HYPOXIA AND METABOLISM

4.1 HIF1: Structure and regulation

In the study of metabolic glycolytic shift, a great interest was given above all to the transcription factor HIF-1α (hypoxia-inducible factor 1α). It is activated in hypoxic conditions and its net effect consists in the increase of glycolysis with reduction of oxidative phosphorylation (Denko, 2008). Indeed, recent studies have demonstrated that the switch from oxidative to glycolytic metabolism is an active response to hypoxia that is mediated by hypoxia-inducible factor 1 (HIF-1). The concept of tumor hypoxia was first proposed in the study of lung cancer tumor tissue (Thomlinson, et al., 1955) . Hypoxia is a common feature of most solid tumors. When the tumor exceeds a certain size and blood vessels are often abnormal or limiting, the inside of the tumor is in anoxic state. Tumor cells in this hypoxic region begin to adapt to these low oxygen tension conditions by activating several survival pathways. Activation of HIF-1 transcription factor is the most recognized pathway adopted by hypoxic cells in this harsh microenvironment. Activated HIF-1 plays a crucial role in adaptive responses of the tumor cells to changes in oxygen levels through transcriptional activation of over 100 downstream genes (approximately 1.0–1.5% of the genome is transcriptionally regulated by hypoxia) which regulate vital biological processes required for tumor survival and progression. Examples include genes involved in cell immortalization and stem cell maintenance, genetic instability, glucose and energy metabolism,vascularization, autocrine growth factor signaling, invasion and metastasis, immune evasion and resistance to chemotherapy and radiation therapy (Semenza, 2010). HIF-1 was initially discovered by Semenza and co-workers in 1991 during a study conducted on erythropoietin (EPO) gene (Semenza, et al., 1991). HIF-1 is a heterodimeric transcription factor, composed of two subunits, the HIF-1α (or its analogs HIF-2α and HIF-3α) and HIF- 1β subunits. HIF-1α is an oxygen sensitive subunit and its expression is induced under hypoxic conditions.

In contrast, HIF-1β is constitutively expressed. The HIF-1α subunit has two transactivation domains (TAD):

- NH2-terminal (N-TAD)
- COOH-terminal(C-TAD).

These two domains are responsible for HIF-1α transcriptional activity (Li, et al., 1996) . C- TAD interacts with co-activators such as CBP/p300 to modulate gene transcription of HIF-1α under hypoxia. N-TAD is responsible for stabilizing HIF-1α against degradation (Yamashita, et al., 2001) **Figure 8**. HIF activity is tightly regulated by cycles of synthesis and oxygen-dependent proteasomal degradation. Indeed, HIFα subunits are continuously synthesized and their stability is regulated by oxygen availability (Gordan, et al., 2007). Under normoxic conditions, HIFα subunits are hydroxylated on proline residues in the oxygen-dependent degradation (ODD) domain by prolyl hydroxylase enzymes (PHD2) and subsequently ubiquitinated by the tumor suppressor protein von Hippel-Lindau (VHL), an E3 Ligase, prior to their degradation in the proteasome (Dang, et al., 2008; Gordan, et al., 2007). Under hypoxic conditions, the reduced molecular oxygen levels decrease the activity of PHDs (Guzy, et al., 2006) consequently, stable HIFα subunits translocate to the nucleus, where it form heterodimers with HIFβ subunits, p300/CBP or SRC-1. Here they bind to specific consensus sequences (hypoxia response element, HRE) in the promoter of hypoxia-responsive genes for the transcriptional activation of the cellular adaptation to hypoxia (Semenza, 2009). In addition to proline hydroxylations, HIF-1 activation is regulated by the hydroxylation of asparagine 803 in the carboxy-terminal transactivation domain (C-TAD) of HIF-1α. This reaction is catalyzed by factor inhibiting HIF-1 (FIH-1). When active under normoxia, FIH-1 decreases the transcriptional activity of HIF-1 by disrupting its interaction with p300/CBP. (Hewitson, et al., 2002) Under hypoxia, FIH-1 is inactivated, thus enabling HIF-1 to become transcriptionally active.

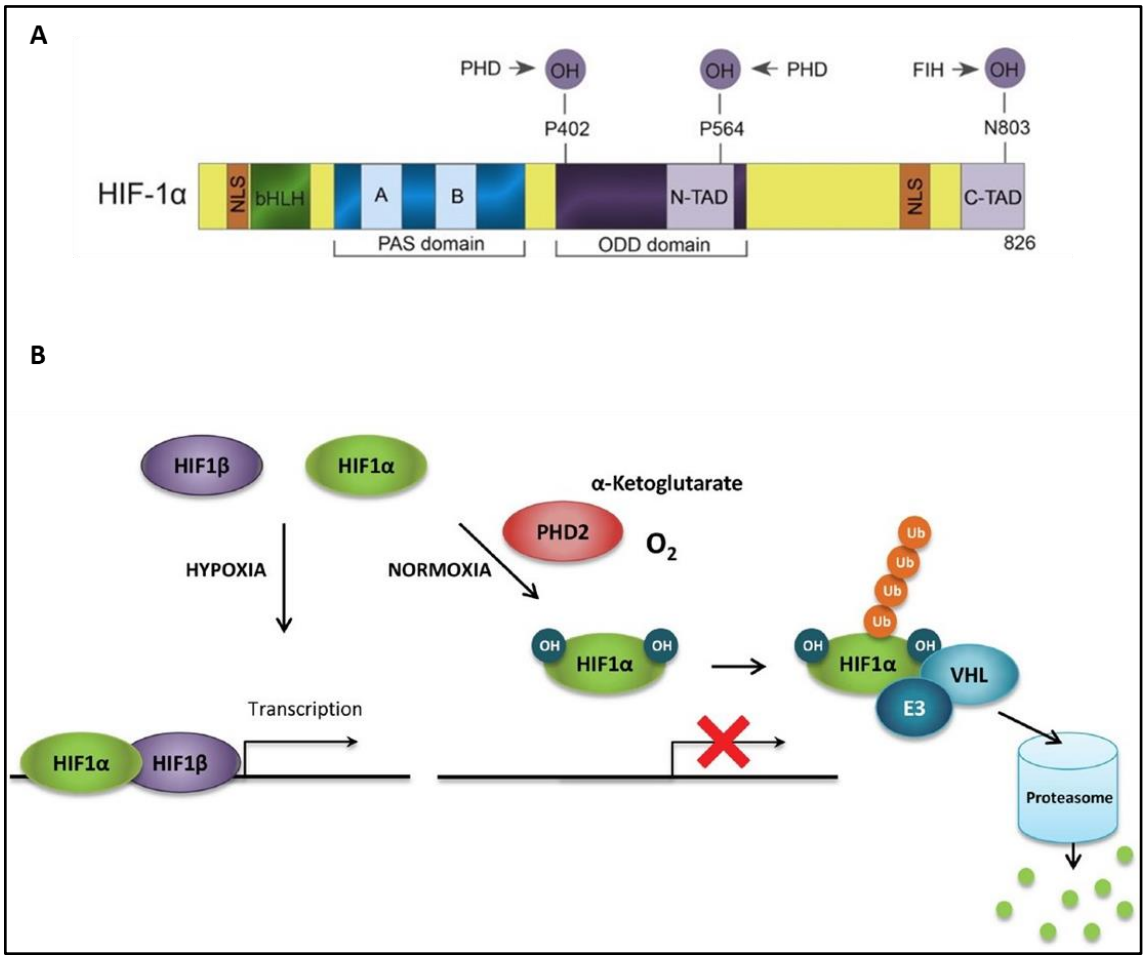


Figure 8 –Structural domains of HIF1α and its regulation. *(A)The HIF1α subunit contain a basic HLH (helix-loop-helix) domain for DNA-specific binding and two sequential PAS (PRE-ARNT-SIM) domains (PAS-A and PAS-B) that facilitate heterodimerization with HIF1β/ARNT. There are two transcription-activation domains (TADs), a N-terminal domain (N-TAD), and one C-terminal domain (C-TAD). The N-TAD is located in the oxygen-dependent degradation (ODD) domain. Conserved residues where PHDs and FIH-1 hydroxylate HIF1-α are specified. Abbreviation: NLS, nuclear localization signal. (B)in the presence of oxygen and α-ketoglutarate, HIF1α subunits are hydroxylated on proline residues in the oxygen-dependent degradation (ODD) domain by prolyl hydroxylases (principally prolyl hydroxylase 2, PHD2). Prolyl hydroxylation is required for the binding of the von Hippel-Lindau protein (VHL), which recruits an ubiquitin ligase complex (E3) that ubiquitinates HIF1α. Ubiquitination marks HIF1α for proteasomal-mediated degradation.*

4.2 HIF-1 target genes involved in glucose and energy metabolism

The HIF-mediated upregulation of glycolysis and suppression of the citric acid (TCA) cycle is a crucial adaptive response at the early stage of hypoxia (Cassavaugh, et al., 2011). It has been documented that HIF-1α activates the expression of glycolytic transporters and glycolytic enzymes to sustain the energy need of cancer cells. GLUT1, GLUT3 (glucose transporter 1, 3) under hypoxic condition ensures sufficient glucose uptake by cancer cells (Gordan, et al., 2007). In addition, the expression of glycolytic enzymes including hexokinases (HK1 and HK2) and phosphoglycerate kinase 1 (PGK1) are also induced by HIF-1α, leading to enhanced glucose flux in glycolysis. The glycolytic enzymes, LDHA (lactate dehydrogenase A) and PDK1 (pyruvate dehydrogenase kinase-1), are also regulated by HIF-1α, they divert the pyruvate flow to lactate differently, in particular: LDHA directly catalyzes the conversion of pyruvate to lactate; PDK1 indirectly negatively regulates the entry of pyruvate into the mitochondria. PDK1 inhibits conversion of pyruvate to acetyl-CoA by phosphorylating pyruvate dehydrogenase (PDH), leading to decreased respiration. In this way the pyruvate is taken away by the TCA and determines consequently the reduction of the electrons's flow through the transport chain. Other genes regulated by HIF1-α and implicated in the metabolic switch and in the survival and growth of the tumors are: MCT4 (monocarboxylate transporter 4) and CAIX (Carbonic anhydrase IX). MCT4 acts to promote conversion of pyruvate to lactate, lactate efflux from the cell and acidification of the cellular environment following the accumulation of lactate. CAIX is an hypoxia-related enzyme expressed on the membrane of cancer cells. CAIX through the conversion of CO2 to bicarbonate and protons, contributes to the maintenance of a pH favorable for tumor cell survival and growth, while also participating in the generation of an increasingly acidic extracellular environment, fueling breakdown of the extracellular matrix and consequent tumor cell invasion (Jamali, et al., 2015). Therefore, CAIX plays a key role in the adaptation of tumor cells to hypoxia. In normal tissue, CAIX expression is absent, but under hypoxic conditions, tumor cells upregulate CAIX in a HIF-1α dependent manner. HIF-1 induces transcription of the PKM gene (Luo, et al., 2011). However, cancer cells replace the

normal form of pyruvate kinase (PKM1) with an alternatively spliced embryonic form (PKM2) which is less active. The switch to PKM2 increases PGAM-1 activity and is again proposed to redirect glycolytic flux away from ATP synthesis and into the production of biosynthetic intermediates (Vander Heiden, et al., 2010,). HIF-mediated expression of vascular endothelial growth factor-A (VEGF-A) and angiopoietin-2 (Ang-2) allow tumour cells to turn around the hypoxic situation by inducing regrowth of the vascular network, a phenomenon termed angiogenesis (Ferrara, et al., 2005). Thereby an oxygenated and nutritional environment is reestablished for maintenance of growth. However, the neo-vessels formed are often distorted and irregular and thus less efficient in oxygen, nutrient transport and drug delivery. Hence the particularly aggressive phenotype of tumors characterized by the development of hypoxic areas. **Figure 9**. However, hypoxia is not the only factor capable of inducing HIF-1α. Recent work has shown that its expression can also be induced under normoxic conditions, through the increased activity of some oncogenes or the reduced function of some oncosuppressors (Bardos, et al., 2004). In particular the HIF system is activated by many tumor suppressor and oncogene pathways, such as:

- Mutation of the von Hippel-Lindau tumor (VHL) suppressor gene (Kaelin, 2008,)

- SRC and ERBB2 kinases are implicated in the reduction of HIF-1α catabolism and, although the mechanism in detail is not yet known, the transcription factor in these cases appears hyperphosphorylated, making it possible to assume a role of oncogenic stress kinases (Jiang, et al., 1997)

- The hyperactivation of the PI3K / Akt / mTORC1 pathway leads to the accumulation of HIF-1α under normoxic conditions (Zundel, et al., 2000) . This phenomenon is more marked in neoplasms in which the alteration of this pathway is an early event (Blancher, et al., 2001). The accumulation can be obtained either through PI3K or Akt activating mutations, or through loss of PTEN function. Furthermore, this accumulation is not mediated by the direct phosphorylation of

HIF-1α by Akt (Zundel, et al., 2000), but is related to a selective increase in the transcription of the HIF-1α gene (Laughner, et al., 2001).

- The RAS/RAF/MAPK pathway has been reported to impact on HIF activity primarily through the regulation of transactivation. Phosphorylation of either HIF-1α or the co-activator p300 by different kinases (either p42/ p44 MAPK or p38) activates HIF, both by promoting the formation of HIF/p300 complexes and by enhancing p300 transactivation (Sang, et al., 2003).

These data demonstrate the strong interconnection between the control points of cell proliferation and survival and those of hypoxic metabolism. Also, HIF-1α seems to be the focal point of this complex network of interactions. For this reason, in the past years several efforts have been made to target this molecule with targeted therapies, with extremely variable results Probably this depends on the existence of many mechanisms in the regulation of HIF-1α which are still unknown. Therefore, it is necessary to understand the role of the proteins that interact with HIF-1α and regulate its activity.

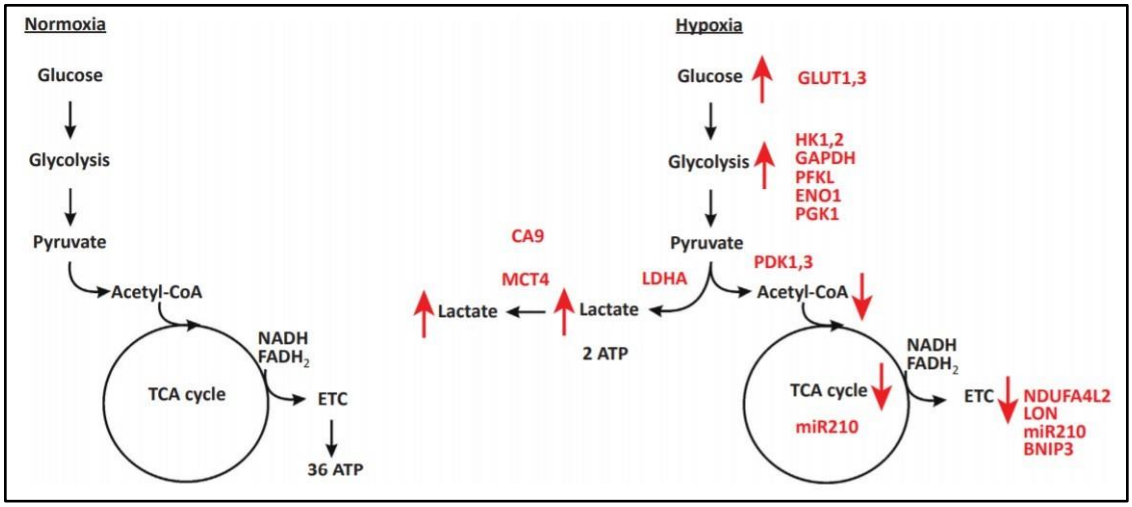


Figure 9 – Reprogramming of energy metabolism by HIF1α. *HIF1α regulates numerous genes involved in glucose uptake and glycolysis.*

5 INTRODUCTION: ORGANOIDS

5.1 The power of a 3D model

Traditional two-dimensional (2D) cell line cultures and patient-derived tumor xenografts (PDTXs) have been employed for long time as tumor models and have made an important contribution to cancer research. However, many drawbacks hamper these two models for clinical use. 2D cell line cultures show their inability in simulating some vital functions, such as the immune system, microenvironment, stromal compartments, and organ-specific functions. Other limitations include the lack of genetic heterogeneity of original tumors after many passages for cancer cell lines (Zhou, et al., 2017). Even PDXs animal models may not suitably reproduce features of human tumors, indeed, the genetic heterogeneity and complexity displayed by human tumors is not reproducible by mouse model (Cheon, et al., 2011; Van Miltenburg, et al., 2012). Organoids technology bridges the conventional 2D in vitro models and in vivo models, and exerts great potential for clinical applications, especially in cancer research. The concept of 3D cell culture has been around for over a century, when Wilson H. V. (1907) demonstrated that mechanically separated cells were capable to differentiate and reorganize, growing into fully functional organisms. Nowadays 3D cell culture has gained a lot of attention and has become increasingly widespread since it can now be applied to mammalian cells. The term “organoid” simply means “resembling an organ”. Organoids are defined by three characteristics: self-organization, multicellularity and functionality (Lancaster, et al., 2014) Fig. 5. Thus, in vitro the cells arrange themselves into the 3-dimensional (3D) organization that is characteristic for the organ in vivo; the resulting structure consists of multiple cell types found in that particular organ and the cells execute at least some of the functions that they normally carry out in that organ. **Figure 10**

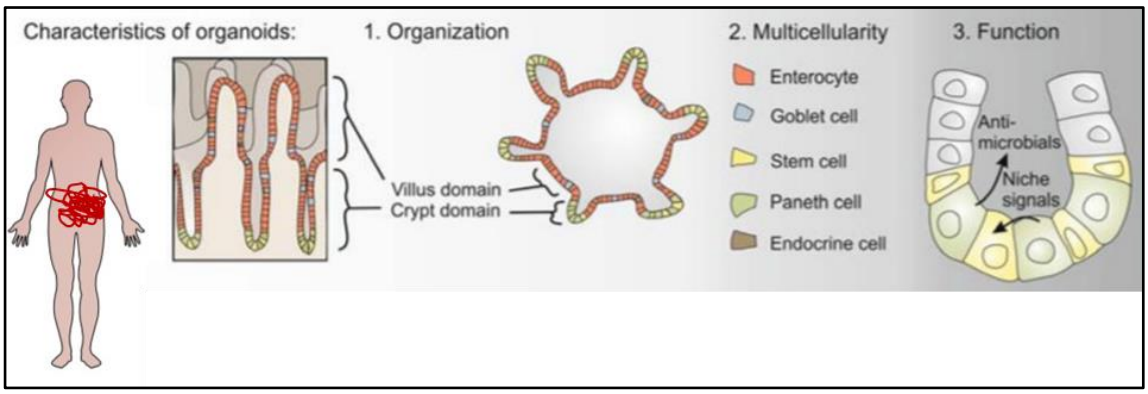


Figure 10 – Organoids are mini-versions of organs. *The definition of an organoid includes the 3 characteristics of organization, multicellularity and function. The example shown here is a small intestinal organoid grown from human adult stem cells. It self-organizes into a 3D structure with small buddings protruding from a central lumen. These buddings contain the cells typically found in the crypts of the small intestine, especially Paneth cells and stem cells. The cystic body contains the cells of the villus region.*

Initially, even 3D spheroids were referred to as organoids, but a recent strict definition of organoids refers to 3D self-organizing cellular structures that recapitulates the properties of the tissue from which the cells are obtained (Aberle, et al., 2018; Clevers, 2016; Drost, et al., 2018). Three-dimensional organoids are different from spheroids since spheroids are suspended cellular aggregates derived from established cell lines and lack any organization (Ishiguro, et al., 2017). Organoids can be derived from two types of stem cells:(i) pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and (ii) organ-specific adult stem cells (ASCs), which are tissue-specific resident stem cells. While ESCs and ASCs are both of natural origin iPSCs are obtained by reprogramming of some specialized adult cells and remarkably are similar to ESCs. iPSCs can be used in specific treatments for each patient, thus avoiding the risk of immune rejection and could potentially overcome ethical issues hampering the development of ESCs for clinical use. First 3D organoid generated and cultured for long time was that of the intestine by Sato et al. (2009), who utilized Matrigel and a cocktail of

growth factors to generate this organoid. The resulting epithelial structures had both proliferative crypt and differentiated villus sections (Sato, et al., 2009). Since the establishment of the first ASC-derived intestinal organoids, the original culture conditions have been tweaked to develop organoids from various mouse and human tissues including the colon, stomach, liver, lung, prostate, pancreas, ovaries, tastebuds (Sato, et al., 2011; Rock, et al., 2009). Potential applications of organoids in research and clinical practice are:

- Tissues morphogenesis & Organogenesis Models
- Tumor, Disease and Infection Models
- Drug Testing
- Toxicity Screening
- Personalized Medicine
- Regenerative Medicine / Organ Replacement

One of the most interesting uses of 3D organoids is the study of tumorigenesis. In this context, 3D organoids are generated from normal tissues. Genes suspected to be associated with cancer development can be edited using CRISPR-Cas9 technology. Other gene manipulation technologies include lentiviral expression vectors to induce the expression of those genes often upregulated in certain cancers (Huang, et al., 2015). Intestinal cancer organoids have been successfully developed in several studies (Vlachogiannis, et al., 2018; Fujii, et al., 2016; Weeber, et al., 2015). Colorectal cancer organoids have been well propagated from different anatomical sites (right-sided, left-sided, and rectal tumors) and rare histological subtypes (mucinous adenocarcinoma and neuroendocrine carcinoma). They showed remarkable resemblance with primary tumors concerning the aspects of histological subtypes, differentiation hierarchies, mutational landscape, and transcriptomic profiling.

6 AIM OF THE PROJECT

TRAP1 is a molecular chaperone with a predominantly mitochondrial localization, overexpressed in numerous tumors and in 50-60% of colorectal carcinomas (Chae, et al., 2012). As explained above, TRAP1 is able to play a regulatory role in numerous cancer cell processes, such as protection from oxidative stress, apoptosis evasion, cell cycle management, and survival under adverse conditions (Kang, et al., 2007; Montesano Gesualdi, et al., 2007; Felts, et al., 2000; Bazzaro, et al., 2006). All these functions are performed by TRAP1 through the quality control of specific client proteins, which are selectively upregulated when TRAP1 pathway is active. The role of TRAP1 as a prognostic factor has recently been elucidated by a study (Maddalena, et al., 2017) which showed that overall survival in colorectal carcinoma is significantly reduced when TRAP1 and its network of client proteins are overexpressed (Matassa, et al., 2012; Landriscina, et al., 2010; Condelli, et al., 2014; Agliarulo, et al., 2015; Lettini, et al., 2016). More recent evidence suggests that TRAP1 is a key regulator of oxidative metabolism, being responsible for the inhibition of complex II and IV of the respiratory chain (Sciacovelli, et al., 2013). Less clear is TRAP1 control of glycolytic pathway and limited data are available about its involvement of metabolic reprogramming under hypoxia. Since TRAP-1 is a protein involved in the regulation of numerous pathways relevant to colorectal carcinogenesis and its clinical importance as a negative prognostic biomarker has been proven, TRAP-1 represents itself a novel interesting therapeutic target. In addition, metabolic alterations, tumor hypoxia and activation of hypoxic signaling pathways has been identified as features strongly associated with aggressive malignancy. The inducible hypoxia factor alpha (HIF-1α) was defined as the key transcription factor that mediates the responses to hypoxia and HIF-1α target genes strongly overlap with those involved in dysregulated tumor metabolism. The expression of several genes is activated following the stabilization of HIF-1α, and this results in a dramatic reprogramming of cancer cell

metabolism involving increased glucose transport into the cell, increased conversion of glucose to pyruvate, and a concomitant decrease in mitochondrial metabolism and mitochondrial mass. For this reason, the aim of this study was to investigate the involvement of TRAP-1 in cellular metabolic remodeling process in human colorectal cancer. In particular, attention has been focused on a key regulator of tumor metabolic rewiring, HIF1α and its interaction with TRAP1 under hypoxia conditions.

7 MATERIALS and METHODS

7.1 Patient Enrollment and PET Analysis

For the study of in vivo glucose uptake, 30 patients suffering from metastatic colorectal cancer were enrolled and subjected to PET global body tomoscintigraphy at the IRCCS CROB of Rionero in Vulture, whose primary tumor was available for further biomolecular analysis. They underwent Positron Emission Tomography (PET) with fluorodeoxyglucose (18F-FDG) to assess glucose uptake from tumor lesions. Expression levels of TRAP1 and GLUT1 were also evaluated on biological samples by immunoblot analysis.

7.2 Cell Cultures and Reagents

Human HCT116 colon carcinoma cells and human cervical carcinoma HeLa cells were purchased from American Type Culture Collection (ATCC) and cultured in McCoy's 5A medium (HCT116) and Dulbecco's modified Eagle's medium (HeLa), supplemented with 10% fetal bovine serum and 1.5 mmol/l glutamine. The HeLa Flp In TRex stable cell lines expressing the eGFP-fusion proteins or the short hairpin RNA (kindly granted by the Prof Franca Esposito, University of Naples) were cultured in the presence of appropriate selective antibiotics. Addition of tetracycline (1 μg/ml) induces proteins as described in (Castello, et al., 2012).

HEK293T cells (human kidney embryonic cell) with high transfectability and transgenic expression were cultured in IMDM Iscove's Modified Dulbecco's Medium supplemented with 10% heat-inactivated FCS, 1% glutamine, and 1% penicillin and streptomycin. This cell line has been used as a packaging cell for the generation of viral particles. The authenticity of the cell lines was verified at the beginning of the project by STR profiling, in accordance with ATCC product description. All cell lines were grown and maintained in a humidified incubator with 5% CO2 and 95% (vol/vol) O2. Hypoxic experiments were performed using the Galaxy 48-R incubator (New Brunswick- an Effendorf company), at

0.5% of oxigen. The hypoxia positive control was obtained by adding Deferoxanime [250μM] into the cell medium.

7.3 Cells Transfection Procedures

TRAP1 transient silencing were performed with siRNA purchased from Qiagen (cat. No° SI00115150). As a control, cells were also transfected with a similar amount of control siRNA (Qiagen, Milano, Italy; cat. no. SI03650318). Transient transfections of siRNAs were performed using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol.

7.4 Organoids

The human p9T colorectal cancer organoids were kindly granted by Professor Jacco van Rheenen during my internship at the department of Molecular Pathology of Netherlands Cancer Institute of Amsterdam. The organoids were cultured in humidified atmosphere at 37°C and 5% CO2 with basal culture medium (Advanced DMEM/F12, 10mM HEPES, 2mM Glutamax, 100 U/ml P/S) supplemented with 10% R-Spondin conditioned medium, 10% Noggin conditioned medium, 1× B27, 1,25 mM n-Acetyl Cysteine, 10 mM Nicotinamide, 500 nM A83-01, 3 uM SB202190. Organoid culture medium was refreshed every two days. To passage the organoids, BME was broken up by pipetting and organoids were collected in a tube. The organoids were centrifuged at 0.4 x rcf for 5 minutes and the medium removed. 2 ml Triple Express was added and the organoids were incubated at 37 ºC for approximately 5 minutes. Every minute, a visual check was done to verify the size or the organoids. Care was taken not to treat the organoids to long with Triple Express. Medium was added and cells were spin down at 0.5 x rcf for 5 minutes. The pellet was taken up in BME and cells were plated in droplets of 5 – 10 uL each. After allowing the BME to solidify, complete medium was added to the plates and organoids were incubated at 37 ºC. To confirm correct sample identity in the lab, used organoids lines were regularly tested by SNP analysis.

7.5 Lentiviral Vectors

Four different validated lentiviral reporter plasmids (pLKO.1_Puro) for shTRAP1 were obtained from the NKI's Robotics and Screening Center facility and have been tested to verify the silencing efficiency induced in colorectal HCT116 cell line and organoids **Figure 11**. Only two of these (shTRAP1 #1 and shTRAP1 #3) were chosen for their high silencing efficiency and reduced mortality. The full hairpin sequences were:

1) shTRAP1 #1:
   CCGGCCGCTACACCCTGCACTATAACTCGAGTTATAGTGCAGGGTGT
   AGCGGTTTTTG;
2) shTRAP1 #3:
   CCGGCAGAGCACTCACCCTACTATGCTCGAGCATAGTAGGGTGAGTG
   CTCTGTTTTTG

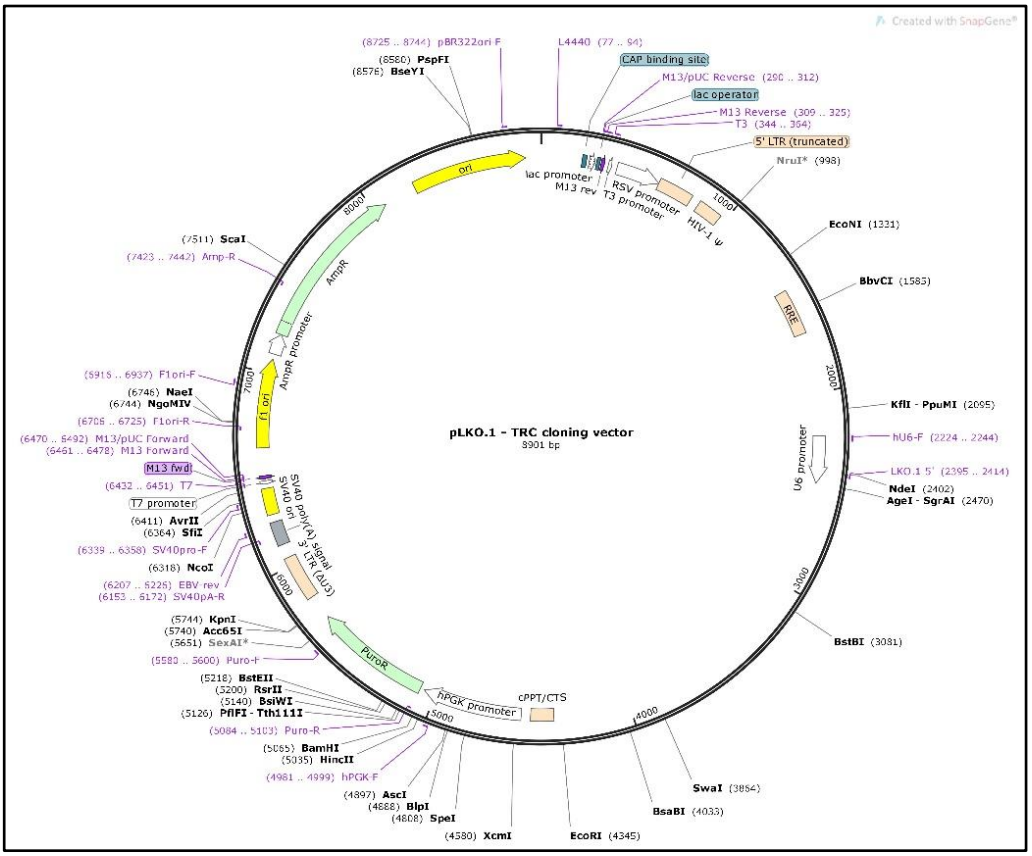


Figure 11 – Lentiviral plasmid map used to produce viral particles

The lentiviral system used is a 3rd generation system that includes:

- *pVSV-G* (Vesicular stomatitis virus G glycoprotein): broad tropism envelope protein used to pseudotype most lentiviral vectors;
- *pMDLg/pRRE*: packaging vector coding for the gag and pol genes of HIV-1, free of all HIV-1 accessory genes (vif, vpu, vpr and nef) used to produce lentivirus recombinants.
- *pREV*: lentiviral vector of packaging coding for the Rev protein of HIV-1.
-

Propagation of AmpR plasmids were performed in the bacterial strain DH5a into LB medium. DNA was isolated using PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen, Cat.number K210004).

7.6 Transient Transfection of HEK293T Cells for Lentiviral Particles Production

HEK293T cells were transfected with pLKO.1 vector coding shTRAP1 using Calcium phosphate-DNA co-precipitation technique. Briefly, on day 0, 293T cells ($7x10^6$) were plated in 150mm dishs in 20 ml of complete medium; the second day two hours before transfection medium was refreshed. Plasmid DNA mix was prepared by adding the packaging plasmids and transfer vector in 0.1x TE/H2O (2:1) (TE Buffer =10 mM Tris-HCl, 1 mM EDTA, pH 8) to a final volume of 1125µl, 125µl of 2.5M of CaCl2 ware then added and the mix left at RT for 5 minutes. Then 1250µl of 2x HBS buffer (NaCl 281 mM, HEPES 100 mM, Na_2HPO_4 1.5 mM in sterile water) were added dropwise while vortexing at maximum speed and immediately added to the HEK293T cells. The cells were exposed to the precipitate for 16 h, after 16 ml of fresh medium were placed, and the cells started to produce viral particles up to a maximum of 30h. The cell supernatant was collected, filtered and directly used for cell transduction, or ultracentrifugated (20,000 rpm) for 2h at 7°C for organoids transduction.

7.7 Organoids Transduction

Lentiviral titers were determined using the qPCR Lentivirus Titration Kit (LV900, abm), following the manufacturer's instructions. For the experiments the amount of lentiviral supernatant used was calculated to achieve the MOI (multiplicity of infection) of 50. To ensure efficient transduction, HEK293T and HCT116 cells were incubated with lentiviral supernatants for 24hrs in the presence of polybrene (8 μg/ml). Antibiotic selection was initiated 24 hr post-transduction and was carried out for five consecutive days. Tumor-derived organoids P9T were e trypsinized using TrypLE to clumps of a few cells. Organoids were mixed with 100 μL concentrated virus, 8 ng/mL Polybrene and 10 μM Rock inhibitor Y-27632 in the 15ml tubes and centrifugated at 600*g* for 1 hour at 32 °C. Subsequently, organoids were incubated at 37 °C and 5% CO2, for 5-6 hours. After incubation, organoids were washed with Advance DMEM/F12 medium and spun down for 5 minutes at 3000 rpm to eliminate the virus. After removal of the virus-containing medium, organoids were plated in approximately 150 μL BME. Transduced organoids were grown in Adavance DMEM/12 full of growth factors + fresh Y-27632 for 3 days after which selection was applied by addition of puromycin (2μg/mL) to the medium for 4 days.

7.8 Immunoblot Analysis

Cell pellets and tumor samples were lysed in ice-cold RIPA buffer (20 mmol/L Tris (pH 7.5) containing 300 mmol/L sucrose, 60 mmol/L KC1, 15 mmol/L NaC1, 5% (v/v) glycerol, 2 mmol/L EDTA, 1% (v/v) Triton X-100, protease and phosphatase inhibitor cocktail and 0.2% (w/v) deoxycholate) for 30 minute at 4°C, homogenized and centrifuged at 12000 rpm for 15 min. according to the manufacturer's procedures. Samples were resolved by *SDS-PAGE* and transferred on nitrocellulose membrane (Bio-Rad Laboratories GmbH, Munchen, Germany). The following antibodies were used: anti-TRAP1 (sc-73604), anti-HIF-1alfa (ab16066 Abcam), MCT4 (sc-376140), GLUT1 (Abcam 32551), PKM (sc-365684), PDK1 (sc-293160), LDHA (sc-137243), Tubulin (sc-5286), Actin(sc-47778). Protein levels were quantified by densitometric analysis using the

Image Lab 5.2.1 software (BioRadLaboratories GmbH). TRAP1 and GLUT1 expression in tumors was considered upregulated if >3 times compared to expression levels in normal non-infiltrated peritumoral mucosa.

7.9 Glucose Uptake Assay

Glucose uptake was assessed using the Glucose Uptake Colorimetric Assay Kit (ab136955, Abcam). The cells were plated at a density of ~ 4000 cells per well in a 96-well sterile plate. Following a pre-incubation of 20 minutes with KRPH (Krebs-Ringer-Phosphate-Hepes buffer containing 20 mM HEPES, 5 mM KH2PO4, 1 mM MgSO4, 1 mM $CaCl_2$, 136 mM NaCl and 4.7 mM KCl, pH 7.4.) / 2% BSA start up glucose carriers. 2-DG 10 mM was added and incubated for 20 minutes, and the enzyme and amplification reaction mixture was added to each well. The measurement of the microplates was performed by reading the optical density at 412nm in kinetic mode, every 2-3 minutes, at 37 ° C, protected from light, according to the manufacturer's protocols. All glucose uptake measurements were performed in duplicate.

7.10 Lactate Production Assay

Lactate production was determined using an L-Lactate Colorimetric Assay kit (ab65331, Abcam) according to the manufacturer's protocols. Briefly the cells were collected in amounts necessary for each test (about 2 $x10^6$ cells), washed with cold PBS and resuspended in the lysis buffer, to be rapidly homogenized and centrifuged. Samples were deproteinate to delete endogenous LDH, and then the reaction mix and lactate enzyme mix were added to each sample. The reaction mixture was incubated for 30 min at room temperature and optical density (OD) values at a wavelength of 450 nm were measured with a microplate reader. All lactate production measurements were performed in duplicate.

7.11 RNA Extraction and Real-Time PCR

Total RNA was extracted from cultured cells by using RNeasy Plus Mini KIT (Quiagen) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically at 260nm using the NanoDrop™ 2000/2000c Spectrophotometer (Thermoscientific). Each RNA was transcribed to cDNA using reverse transcription reagents (Superscript IV Vilo, Invitrogen) according to the manufacturer's instructions. The mRNA expression levels of GLUT1, CAXI, MCT4, PDK1, LDHA, PKM, VEGFa and TRAP1 were measured by quantitative real time PCR in The CFX96 Touch™ Real-Time PCR Detection System (BiorRad) using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) according to the manufacturer's instructions. The following used primers are validated and purchased by Sigma-Aldrich:

GLUT1: forward 5'-AGTTCTACAACCAGACATGG-3', reverse 5'-CAGGTTCATCATCAGCATTG-3'; *CAIX*: forward 5'-GAAGAAATCGCTGAGGAAG-3', reverse 5'-GAGACCCCTCATATTGGAAG-3'; *MCT4*: forward 5'-GGAGCCCTTATATTATTTGGAG-3', reverse 5'-GTAGACTCTTCTGTCTCATGG-3'; *PDK1*: forward 5'-ATGATGTCATTCCCACAATG-3', reverse 5'-AAGAGTGCTGATTGAGTAAC-3'; *LDHA* : forward 5'-CACCATGATTAAGGGTCTTTAC-3', reverse 5'-AGGTCTGAGATTCCATTCTG-3'; *PKM2*: forward 5'-ATGTTGATATGGTGTTTGCG-3', reverse 5'-ATTTCATCAAACCTCCGAAC-3'; *VEGFa* : forward 5'-ACATAGGAGAGATGAGCTTC-3', reverse 5'-CTTATACCGGGATTTCTTGC-3'; *TRAP1*: forward 5'-CGCAGCATCTTCTACGTGC-3', reverse 5'-CTGATGAGTGCGCTCTCC-3'; *α-Tubulina*: forward 5'-CTTTGTATTTGGTCAGTCTGG-3', reverse 5'-ATCTTGCTGATAAGGAGAGTG-3', *β-actin*: forward 5'-GACGACATGGAGAAAATCTG-3', reverse 5'-ATGATCTGGGTCATCTTCTC-3'

Reaction conditions were as follows: preincubation at 95°C for 5 min, followed by 45 cycles of 10 sec at 95°C, 10 sec at 60°C, and 10 sec at 72°C. β-actin and α-tubulin were

chosen as the internal control. In PCR analyses performed upon TRAP1 silencing, RNAs were collected 72 h after siRNA transfection.

7.12 Gene Expression Profiles

For each sample, 300ng of total RNA were transcribed for the synthesis of cDNA and biotinylated cRNA according to the protocol of the *Illumina TotalPrep RNA* (Ambion) amplification kit. The hybridization, marking and scanning of 750ng of cRNA were performed on the *Illumina Human HT12 v4.0 Expression BeadChip* array (Illumina Inc.), following the standard protocol. All analyzes were done in triplicate for each sample. The BeadChip was then dried and scanned with the *Illumina HiScanHQ system* (Illumina Inc.). The data analysis was carried out through the free/open source environment *R/Bioconductor*. The probes with a low quality of the fluorescence intensity signal were excluded from the subsequent analysis steps. Normalization was performed using the neqc procedure, a background correction through internal controls followed by *Quantile Normalization*. The differential analysis between the conditions (groups) was processed thanks to the *R/Bioconductor limma* package; the method of statistical correction for multiple comparisons applied is Benjamini-Hochberg, obtaining an adjusted p-value. The functional enrichment was carried out through the *GSEA* computational method and the use of the MsigDB Database for the collection of annotated gene sets, in particular we focused on the following categories: Hallmark, Gene Ontology and Pathways.

7.13 Statistics

The paired Student's *t* test was used for the analysis of metabolic tests in silenced/transfected/treated cells and related controls. Data are reported as mean value of least 3 independent experiments (± SD). The Spearman test was used to establish statistical significance of TRAP1, GLUT1 and SUV (standardized uptake value) correlation in human CRCs. Real-Time data were analyzed by analysis of variance (ANOVA) and appropriate post-test (GraphPad 7.0 software).

8 RESULTS

As discussed above, one of the main metabolic effects of TRAP1 is the suppression of oxidative phosphorylation at mitochondrial level by inhibition of the respiratory chain complex II (Yoshida, et al., 2013; Sciacovelli, et al., 2013). This data suggests that tumors with high TRAP1 expression are likely characterized by enhanced glycolytic metabolism, this representing the starting point to study the role of TRAP1 in hypoxia-induced metabolic rewiring in colorectal carcinoma.

8.1 TRAP1 is Correlated with GLUT1 Expression and Glucose Uptake Upon ^{18}F-FDG PET Study in Human Colorectal Carcinomas

To study the role of TRAP1 in Warburg's metabolism, the expression of TRAP1 and GLUT1 was analyzed in 26 patients affected by metastatic colorectal carcinoma, studied at clinical level by Positron Emission Tomography (PET) with the administration of fluorodeoxyglucose (^{18}F-FDG). The analysis of the Standardized Uptake Value (SUV), an index of fluorodeoxyglucose uptake, confirmed that most of the lesions were positive. The ^{18}F-FDG SUV index was correlated with the expression of TRAP1, quantified by immunoblot on the primary tumor. TRAP1 was upregulated in 73% of primary CRCs, whereas GLUT1 was overexpressed in 65% of cases **Figure 12**.

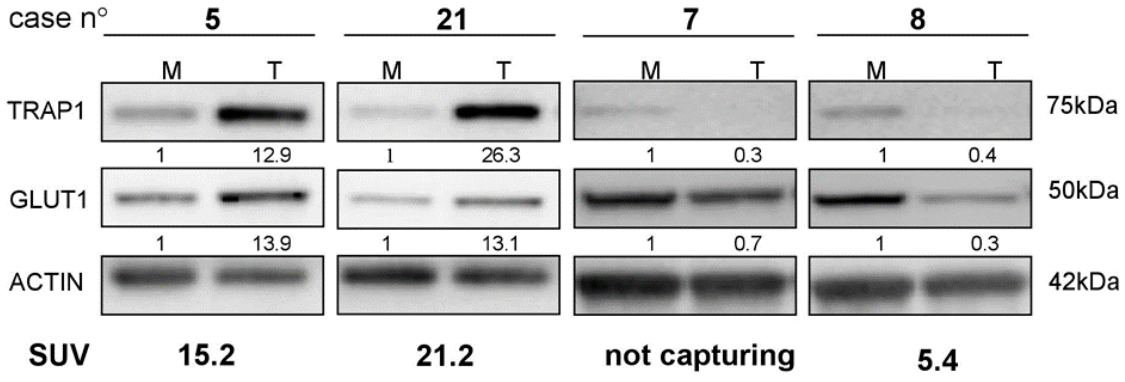


Figure 12 – Correlation between TRAP1 e GLUT1. *TRAP1 and GLUT1 protein levels in four representative cases of human colorectal carcinomas (T) and respective peritumoral non-infiltrated mucosas (M) by immunoblot analysis*

Statistical analysis demonstrated a direct and statistically significant correlation between the expression of TRAP1 and the SUV (Spearman: 0.479; p-value: 0.013 **Figure 13**).

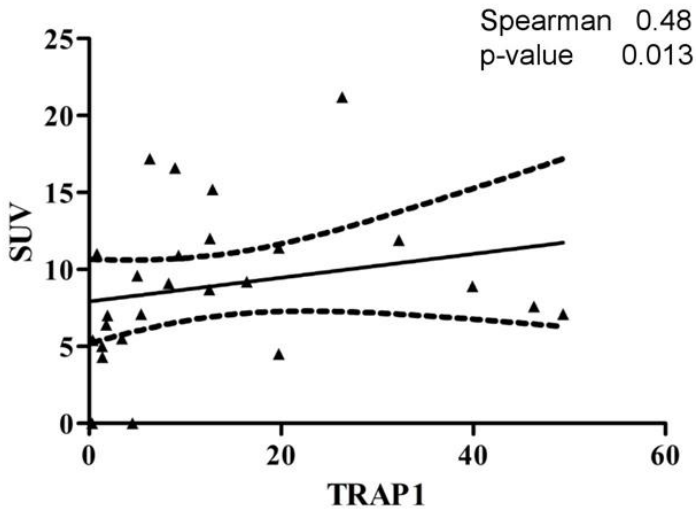


Figure 13 – Correlation between TRAP1 and glycolytic metabolism in human metastatic colorectal carcinomas. *Statistical correlation between TRAP1 and ^{18}F-FDG uptake by Positron Emission Tomography in the same tumor samples.*

Furthermore, the expression levels of TRAP1 and GLUT1 proteins showed a direct and statistically significant correlation (Spearman: 0.995; p-value: 3.581e-0.14 **Figure 14**).

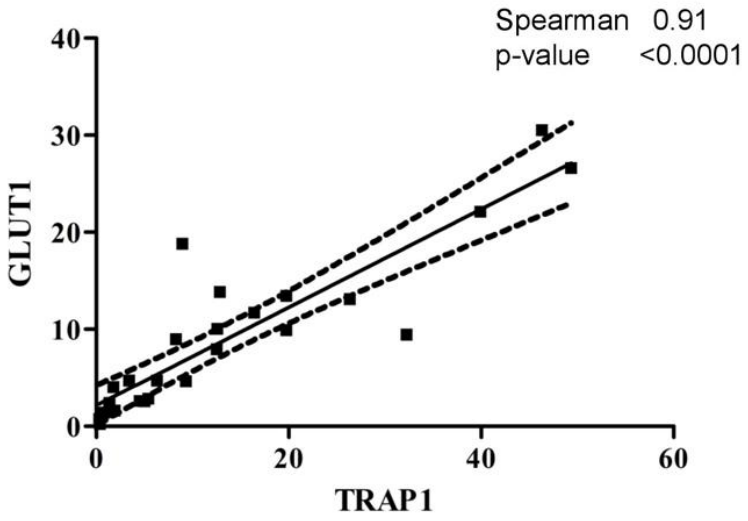


Figure 14 – Correlation between TRAP1 and GLUT1 expression levels in human metastatic colorectal carcinomas. *Statistical correlation between TRAP1 and GLUT1 in 26 patients with colorectal carcinoma, subjected to PET.*

These data indicate that colorectal tumors with high TRAP1 expression are characterized by higher GLUT1 expression and greater glucose uptake.

8.2 TRAP1 Regulates the Glycolytic and Oxidative Metabolism in Human Colorectal Carcinoma Cell line

To support the results obtained in vivo, the study was further performed in HCT116 colorectal cancer cells, transiently silenced for TRAP1 and analyzed for glucose uptake, lactate production, oxygen consumption rate (OCR) and GLUT1 expression. The glucose uptake assay shows how the internalization of 2-DG (molecular analogue of glucose) is significantly reduced more than 40% (p-value: 0.0001) when TRAP1 is silenced compared to control cells **Figure 15A**. Furthermore, under the same experimental condition, a significant reduction of lactate production by 80% (p-value: 0.015) was observed **Figure 15A**. Consistently with metabolic assays, GLUT1 expression level was significantly reduced in the immunoblot analysis compared to control cells (**Figure 15A**, insert). In addition, lactate production and mitochondrial oxygen consumption rate (OCR) were evaluated in multiple

experiments with different efficiency of TRAP1 expression. Of note, a direct correlation was observed between high TRAP1 expression and lactate production and an inverse correlation between TRAP1 and cellular respiration **Figure 15B**, with OCR progressively increased upon TRAP1 downregulation.

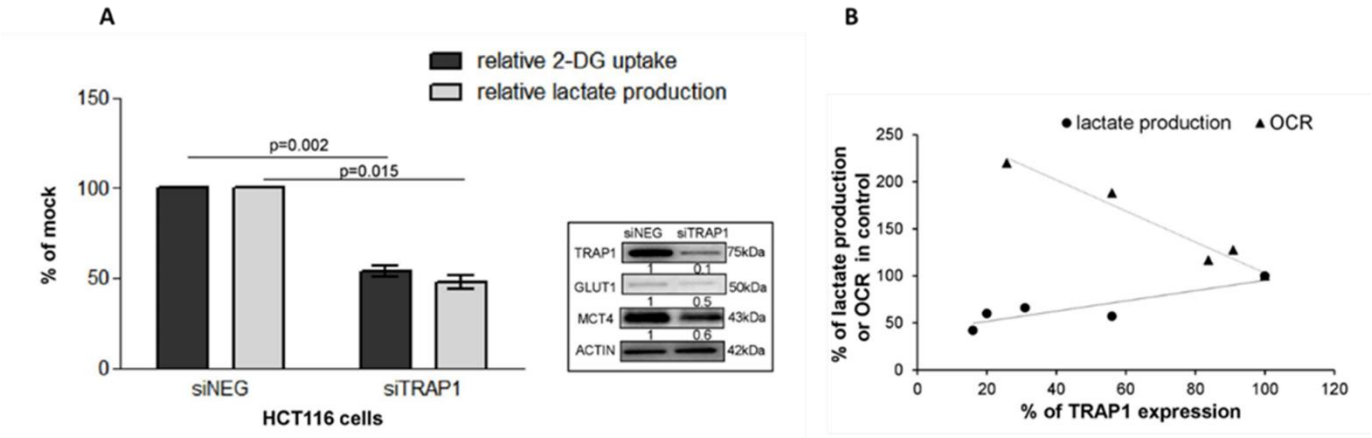


Figure 15 – Metabolic assay in vitro *(A) 2-DG uptake and lactate production in TRAP1-silenced HCT116 cells; insert: immunoblot analysis of TRAP1, GLUT1 and MCT4 under the same experimental conditions (B) relationship between TRAP1 expression and mitochondrial oxygen consumption rate (OCR) or lactate production.*

These data support the role of TRAP1 in regulating both glucose internalization and its consumption to balance the inhibition of mitochondrial respiration.

8.3 HIF1-alfa Stabilization is Mediated by TRAP1 Expression Levels in Colorectal Cancer Cell line

As previously underlined, cancer cells are able to survive in a hypoxic environment and hypoxia is the main stimulus for the activation of glycolytic metabolism mediated by HIF-1α. Thus, subsequent experiments were aimed at demonstrating a possible role of TRAP1 in controlling the activation of HIF-1α. HCT116 colorectal cancer cells were used to generate a hypoxic cellular model for these experiments. Preliminary tests were done to evaluate the optimal hypoxia condition, both in terms of oxygen percentages (0.5%) and exposure times that allow an induction of HIF-1α. As reported in **Figure 16**, HIF-1

αexpression becomes evident upon 2h and 4h cell exposure to 0.5% O2. As a control for these experiments, cells were treated with deferoxamine (positive control of HIF-1α induction), with evidence of HIF-1α stabilization.

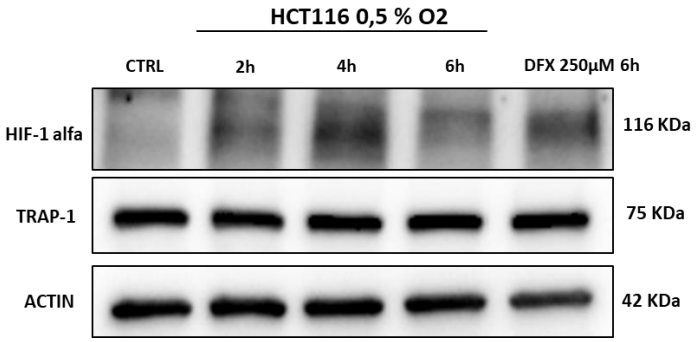


Figure 16 – HIF-1α stabilization. *Maximum induction of HIF-1α after 2 hours and 4 hours of incubation at 0.5% of oxygen.*

In subsequent experiments, HCT116 cells were exposed to hypoxia after knocking down TRAP1 expression by siRNA. Interestingly, in the same time interval, the stabilization of HIF-1α expression was partially inhibited in conditions of TRAP1 silencing **Figure 17A**. To support this observation, Hela cells, transfected with a TRAP1 inducible vector, were exposed to hypoxia. Noteworthy, TRAP1 overexpessing cells exhibited HIF-1α stabilization under normoxic conditions and further enhancement of its levels upon exposure to hypoxia **Figure 17B**

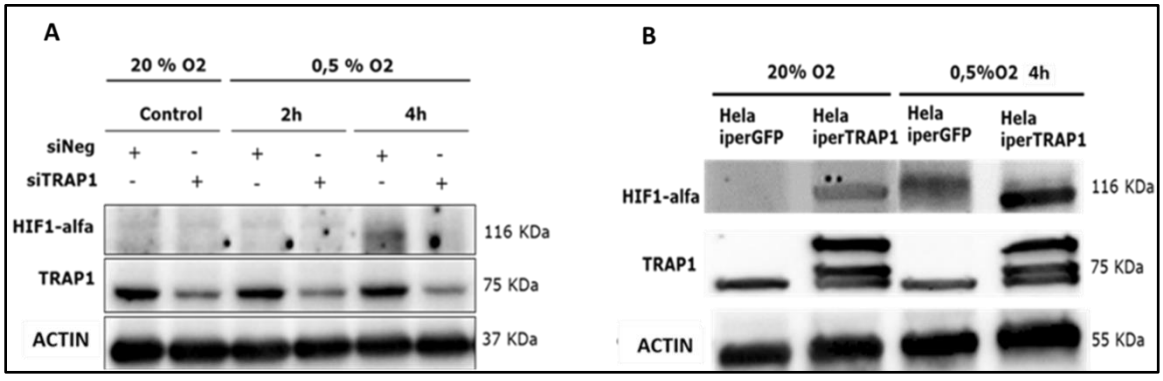


Figure 17 – TRAP1 expression levels influence HIF-1α stabilization. *(A) HCT116 cells transiently silenced for TRAP1 exposed to 0,5% of oxygen for 2h and 4h; (B) Hela TRAP1 inducible cells exposed to hypoxia (0.5 % O2) for 4h.*

These data allow us to hypothesize that TRAP1 metabolic control can be mediated by its ability to modulate cellular response to hypoxia through HIF-1α stabilization.

8.4 TRAP1 Regulates Glycolytic Metabolism in Conditions of Oxygen Deprivation

Metabolic experiments were also performed under hypoxia. HCT116 cells transiently silenced for TRAP1 were incubated at 0.5% O2 for 4h and analyzed for glucose uptake and lactate production. Data show that hypoxia highly enhanced 2-DG uptake in control cells, while TRAP1-silenced cells were unable to upregulate 2-DG uptake (p-value: 0.02) **Figure 18A**. Likewise, lactate production was significantly enhanced in control cells, and only moderately in TRAP1-silenced cells (p-value: 0.04) **Figure 18B**. Immunoblot analysis confirmed that the TRAP1 silencing leads to a downregulation of GLUT1 expression in both normoxic and hypoxic conditions **Figure 18C**.

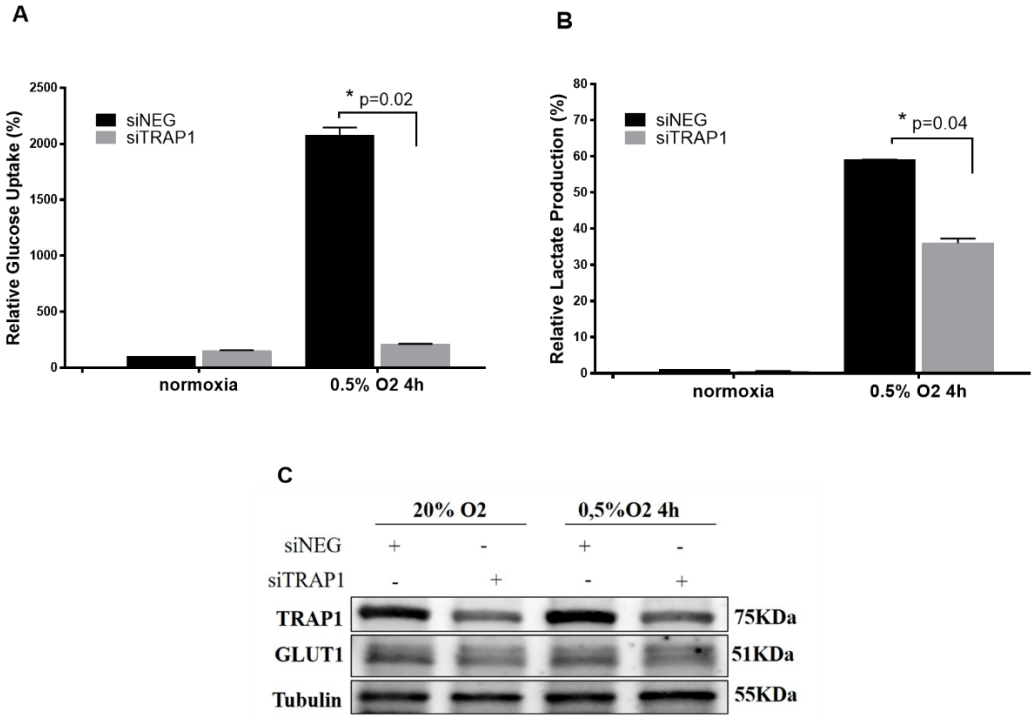
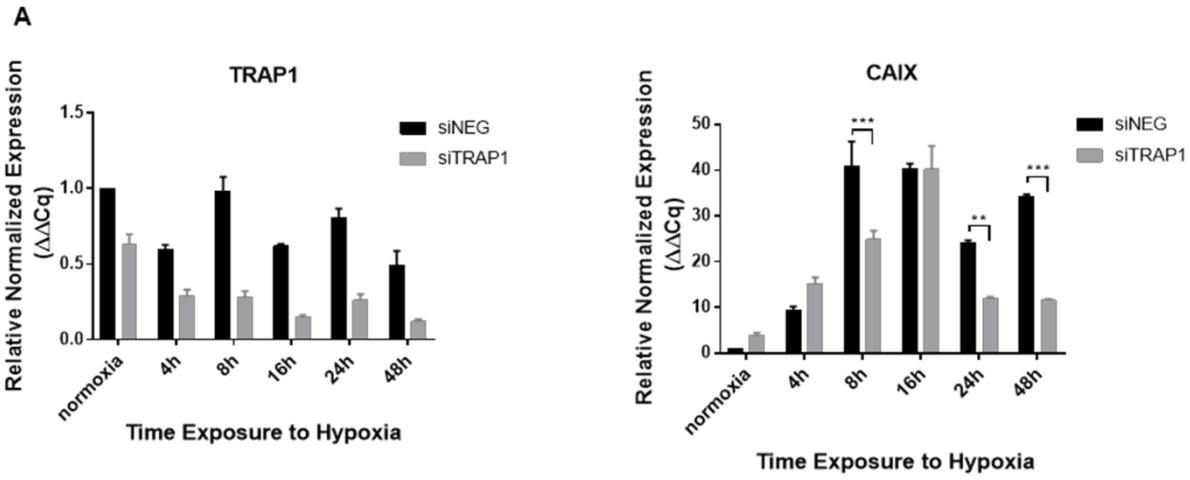


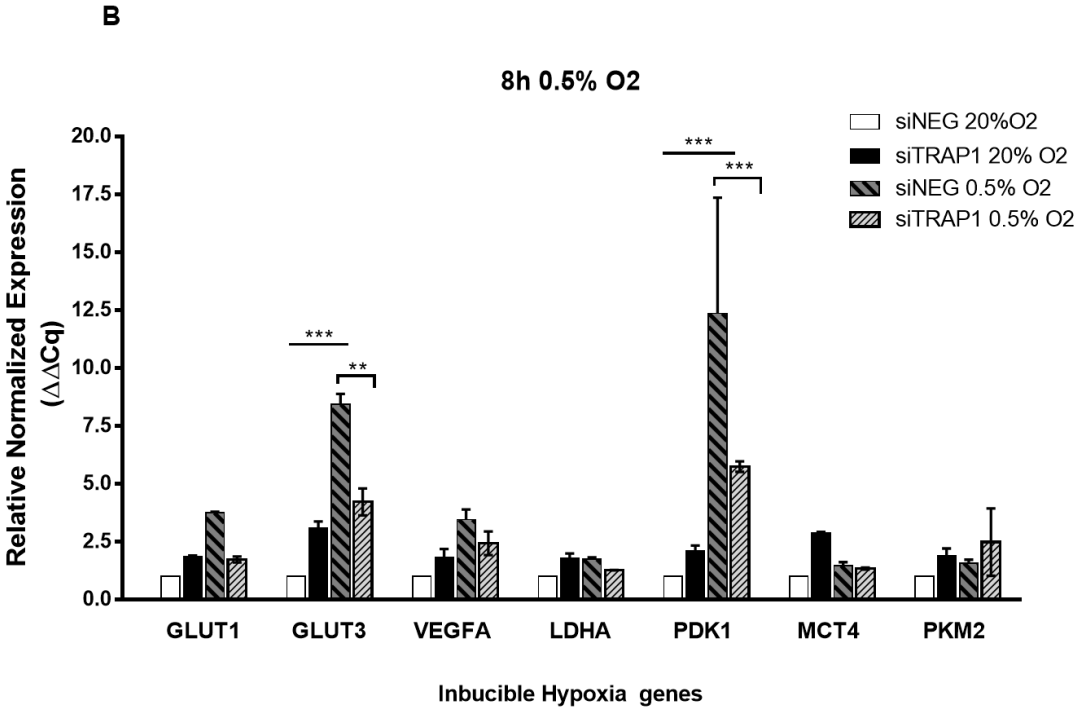
Figure 18 – Metabolic assay under hypoxia *(A, B) Relative glucose uptake and lactate production in HCT116 TRAP1 transiently silenced cells exposed to 0,5% of O2 for 4h compared to negative control (siNEG). Statistical significance is indicated with (*) p< .05 (B) Immunoblot analysis of TRAP1, and GLUT1.*

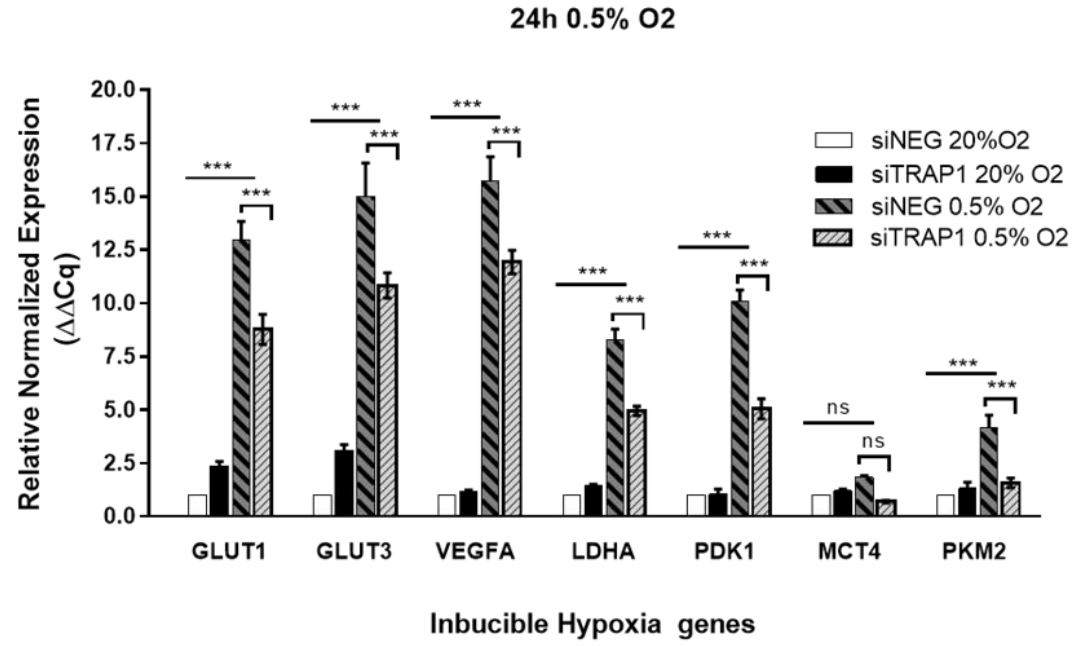
8.5 TRAP1 Affects the Metabolic Transcriptional Activity Induced by Hypoxia

In order to characterize the role of TRAP1 in metabolic rewiring under hypoxic conditions in colorectal carcinoma, we decided to study the expression of genes modulated by the transcriptional factor HIF1α in a time course experiment. HCT116 cells, transiently silenced for TRAP1, were incubated at 20 and 0.5% of O2 for 4h, 8h, 16h, 24h and 48h. Since in normal tissue, CAIX expression is absent, while is induced under hypoxic conditions in a HIF-1α-dependent manner (Jamali, et al., 2015), we used CAIX as positive marker of HIF-1α stabilization. Cells exposed to oxygen deprivation exhibited CAIX upregulation compared with normoxic controls. At 8h, 24h and 48h of hypoxia, the silencing of TRAP1 resulted in a significant reduced upregulation of the transporter, with

a significant reduction at 8h (p-value <0.001), at 24h (p-value= 0.002) and at 48h (p-value< 0.001) **Figure 19A**. Consistently with modulation of CAIX expression levels at 8h, 24h and 48h of hypoxia, other HIF-1α inducible genes showed a reduced upregulation following TRAP1 silencing **Figure 19B**. In particular, HCT116 cells transiently silenced for TRAP1 and incubated for 8h at 0.5% of O2 failed to fully activate the expression of GLUT3 (p-value <0.01) and PDK1 (p-value <0.001). At 8h of hypoxia, GLUT1, VEGFA, MCT4, PKM2 and LDHA did not show any significant modulation in relation to oxygen deprivation and the presence/absence of TRAP1. At 24h and 48h of hypoxia, all genes showed a significant negative regulation upon TRAP1 silencing compared with control cells (p-value <0.001), except for MCT4 at 24h (p= ns). Under normoxia, no differences were observed between control and siTRAP1 cells at all time points (data not shown), with low and similar expression levels of all tested genes.




B
8h 0.5% O2
siNEG 20%O2
siTRAP1 20% O2
siNEG 0.5% O2
siTRAP1 0.5% O2
Relative Normalized Expression (ΔΔCq)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
***
**
GLUT1
GLUT3
VEGFA
LDHA
PDK1
MCT4
PKM2
Inbucible Hypoxia genes


24h 0.5% O2
siNEG 20%O2
siTRAP1 20% O2
siNEG 0.5% O2
siTRAP1 0.5% O2
Relative Normalized Expression (ΔΔCq)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
***
ns
GLUT1
GLUT3
VEGFA
LDHA
PDK1
MCT4
PKM2
Inbucible Hypoxia genes

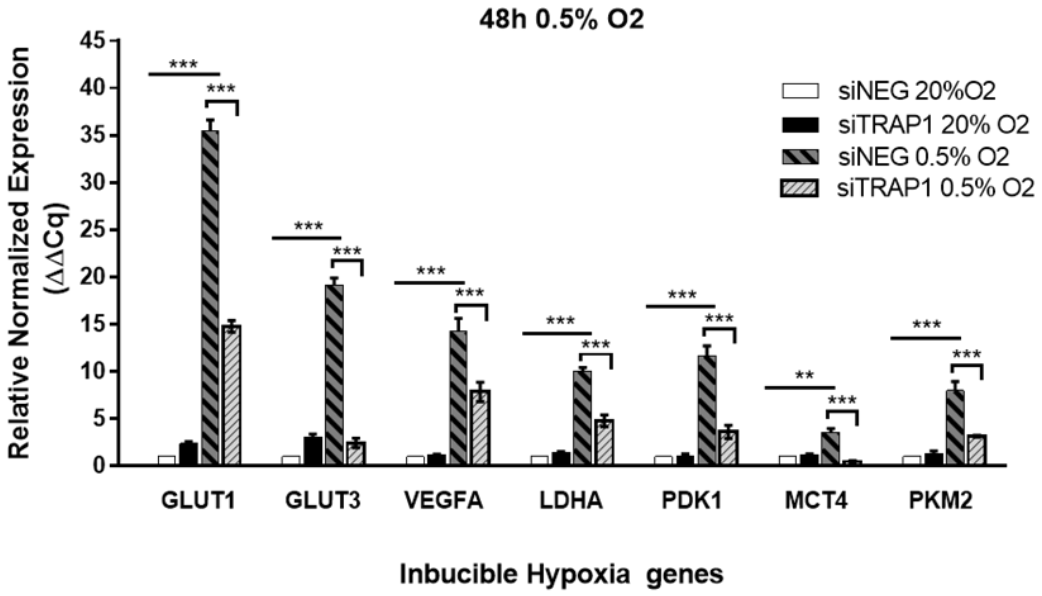


Figure 19 – HIF-1α inducible gene expression in HCT116 control and siTRAP1 cells. *(A) Cells were exposed to hypoxia (0.5%O2) or normoxia (20% O2) for 4h, 8h, 16h, 24h and 48h. Realtive mRNA expression levels for TRAP1 and CAIX at all time points. (B) The expression of the inducible HIF-1α genes are shown only at time points with reduced up-regulation of CAIX in hypoxia following silencing of TRAP1. Quantitative RT-PCR was carried out on extracted mRNA using primers for GLUT1, GLUT3, VEGFA, LDHA, PDK1, MCT4 and PKM2. Ordinate values are based on standardization of target gene expression to that of α-Tubulin and β-actin. Statistical significance is indicated with (*)p< 0.05; (**); p< 0.01; (***) p< 0.001 by comparing siNEG 0.5%O2 vs siNEG 20%O2, and siTRAP1 0.5% O2 Vs siNEG 0.5% O2.*

We also permormed an immuno-blot analysis on the same samples from TRAP1-silenced HCT116 cells incubated for 24 and 48h at 20 and 0.5% of O2 **Figure 20**. TRAP1 downregulation under hypoxic condition leaded to lower stabilization of HIF-1α compared to control cells, and this correlated with the parallel lower upregulation of HIF-1α targets, GLUT1, LDHA and MCT4.

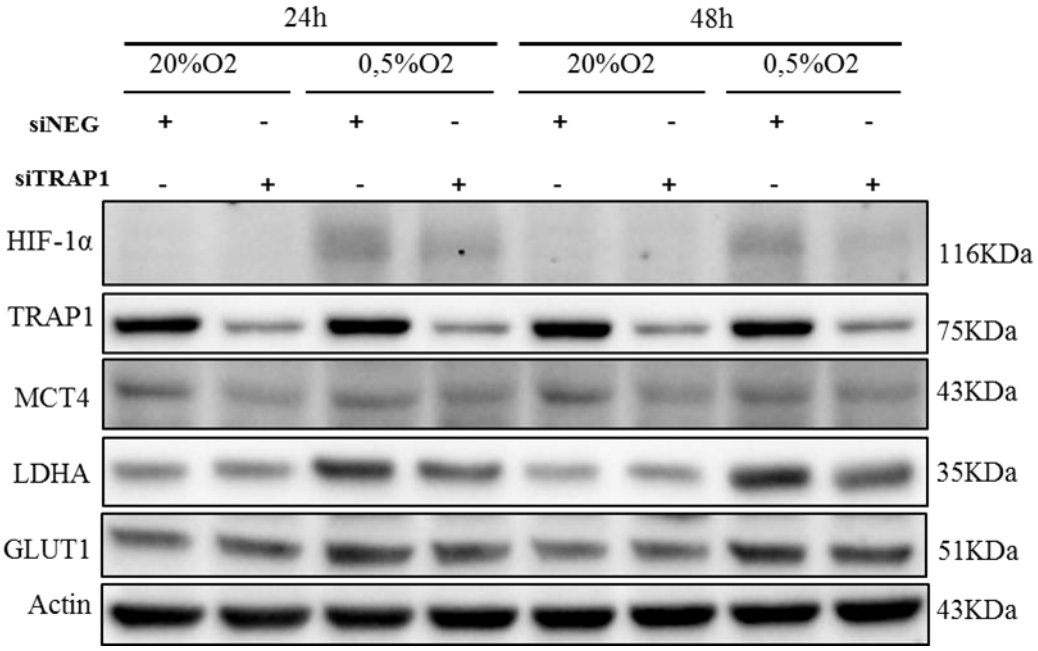


Figure 20 – Control and TRAP1-silenced HCT116 exposed to 20 and 0.5% O2 for 24h and 48h. *Immuno-blot analysis of TRAP1, HIF-1α, MCT4, LDHA and GLUT1. β-actin was used as housekeeping gene.*

Based on these results, it is possible to hypothesize that TRAP1 is involved in the regulation of the characteristic metabolic and gene expression remodeling of hypoxic tumor cells. Thus, we decided to set up a whole genome gene expression profiling of HCT116 cells silenced for TRAP1 and exposed for 24h and 48h at 0.5% of O2in order to have a deeper view of pathways and biofunctions that are activated in condition of limited oxygen availability in colorectal carcinoma cells.

8.6 TRAP1 Prevents the Activation of Hypoxia-Induced Pathways

Previous data suggest that TRAP1 silenced cells are characterized by an altered response to oxygen deprivation in terms of transcriptional activity, with the upregulation of several HIF-1α inducible genes significantly impaired. For this reason, we decided to perform a whole genome gene expression profiling in TRAP1-silenced and control HCT116 cells exposed to normoxia and hypoxia for 24 and 48h. Three different biological replicates of siTRAP1 HCT116 cells and control cells were used. TRAP1 was found to be silenced for

about 60% in each triplicate at both time points. In addition, under hypoxia, the expression of HIF-1α was appreciate **Figure 21 A-B**.

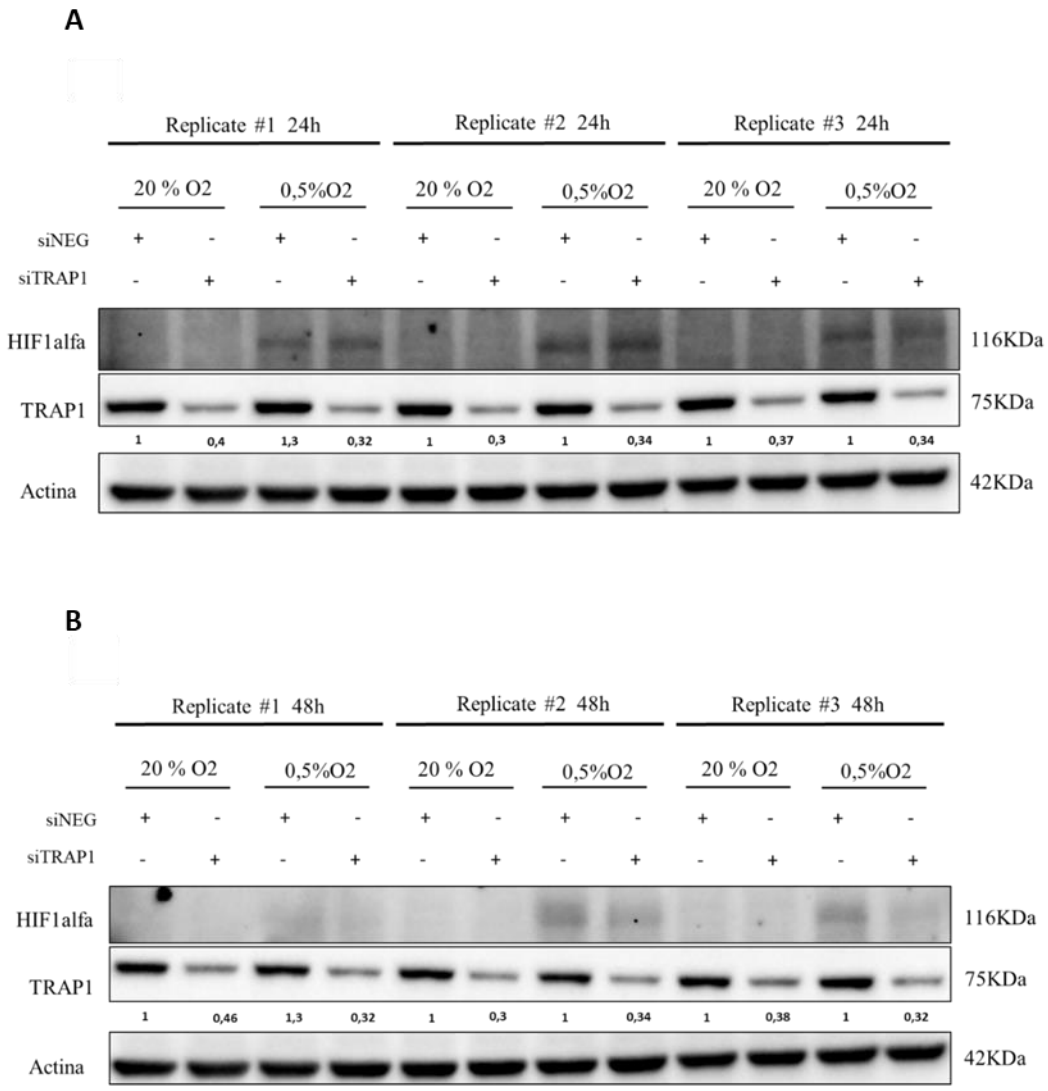


Figure 21 – Control of experimental conditions. *TRAP1 silencing and HIF-1α at 24h (A) and at 48h (B) of hypoxia.*

As expected, the exposure of HCT116 cells to hypoxia resulted in a significant reprogramming of gene expression with 34 and 47 genes downregulated and 45 and 36 genes upregulated at, respectively 24 and 48h. In order to understand the contribution of TRAP1 to this hypoxia-induced gene expression remodeling, GSEA enrichment analysis

was performed to highlight hallmarks that are differentially modulated in control and siTRAP1 cell lines exposed to hypoxia. The heatmap in Figure 22 reproduces the ES values (GSEA enrichment scores) for statistically significant hallmarks as obtained in each individual condition.

A hierarchy clustering analysis allowed to classify our samples in 4 clusters:

- siNEG and siTRAP1 under normoxia at 24 hours (colums 1,2,5 and colums 3,4,6 respectively);
- siNEG and siTRAP1 under normoxia at 48h (columns 7,8,11 and columns 9,10,12, respectively);
- siNEG and siTRAP1under hypoxia at 24 hours (columns 16,17,18 and columns 13,14,15, respectively);
- siNEG and siTRAP1 under hypoxia at 48 hours (columns 19,20,21 and columns 22,23,24, rispectively).

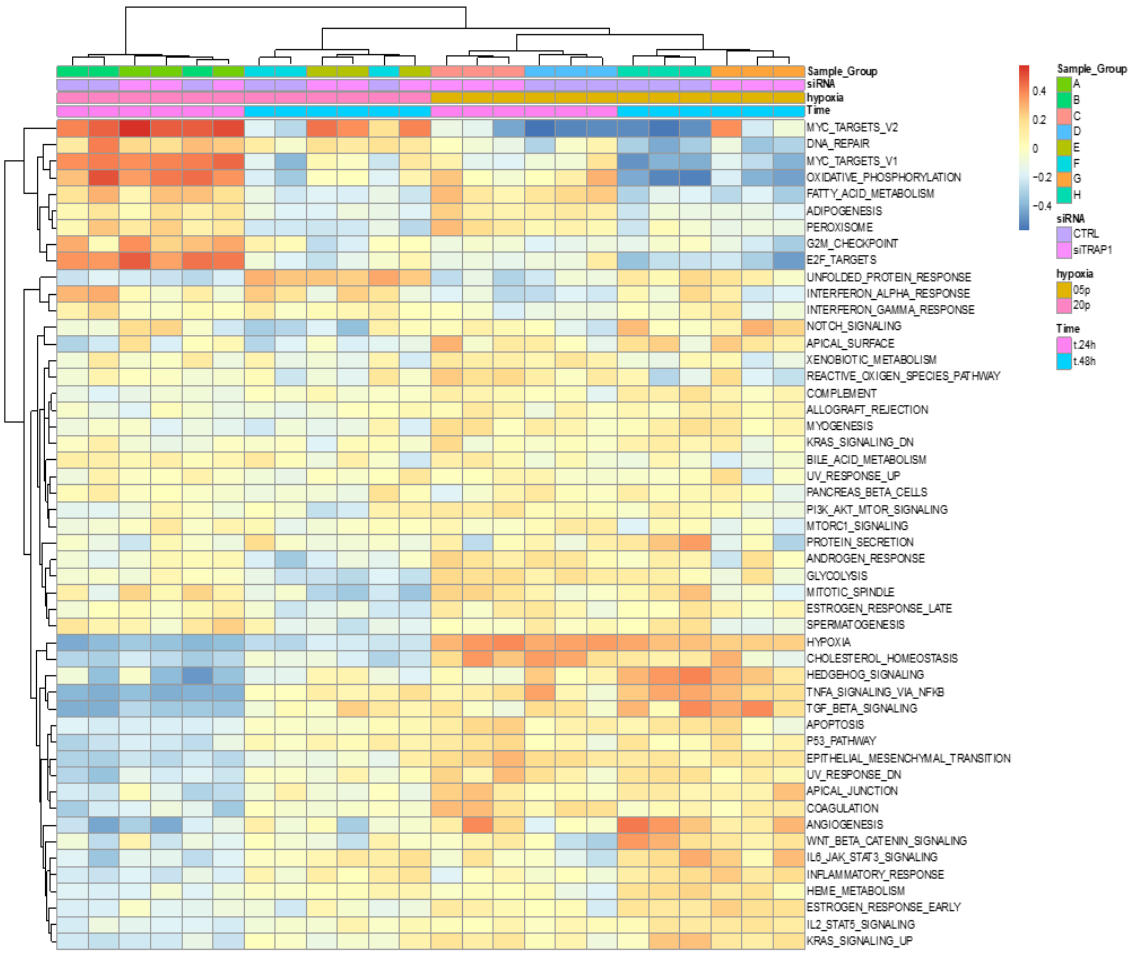


Figure 22 – Heatmap of ES values for statistically significant hallmarks in each experimental condition. *Sample groups: A) siTRAP1 24h 20% O2; B) siNeg 24h 20% O2; C) siTRAP1 24h 0.5% O2; D) siNeg 24h 0.5% O2; E) siTRAP1 48h 20% O2; F) siNeg 48h 20% O2; G) siTRAP1 48h 0.5% O2; H) siNeg 48h 0.5% O2*

As expected, the hallmark of hypoxia was significantly upregulated in control cells exposed to hypoxia compared to normoxia conditions. More specifically, in TRAP1 expressing cells the hallmark was upregulated at both 24h (nes= 2.67; pvalue= 0.001) and 48 hours (nes= 2.85; pvalue= 0.001) upon hypoxia **Table 5A**. By contrast, TRAP1 silencing favored a slight upregulation of the hallmark in normoxic conditions at 24h (nes=1.51; pvalue= 0.001) and at 48h (nes= 1.47; pvalue= 0.001) and a reversal of its activation in

hypoxia conditions at 24h (nes= -1.33; pvalue= 0.019) and at 48h (nes= -1.61; pvalue= 0.0005). This observation further support the role of TRAP1 in the cellular response to hypoxia **Table 5B**.

A

Into siNEG				0,5%Vs20% 24hrs					0,5%Vs20% 48hrs				
Row.Names	adj.Fisher SumLogs	Fisher SumLogs	N.pvalue Signif	Es	pvalue	Nes	N.leading EdgeSubse	N.geneSet	Es	pvalue	Nes	N.leading EdgeSubse	N.geneSet
HALLMARK HYPOXIA	2,27876E-	3,19026E-08	4	0,8136202	0,001	2,6733	104	191	0,7847496	0,001	2,848745	88	191

B

siTRAP1 Vs siNEG				24hrs 20%O2					24hrs 0,5%O2				
Row.Names	adj.Fisher	Fisher	N.pvalue	Es	pvalue	Nes	N.leading	N.geneSet	Es	pvalue	Nes	N.leading	N.geneSet
Hallmark HYPOXIA	7,29859E0	2,91943E-08	4	0,35852	0,001	1,513404	45	191	-0,36191	0,019	-1,32891	34	191
				48hrs 20%O2					48hrs 0,5%O2				
				Es	pvalue	Nes	N.leading	N.geneSet	Es	pvalue	Nes	N.leading	N.geneSet
				0,357488	0,001	1,47533	36	191	-0,40198	0,0005	-1,61557	47	191

Table 5 – Hypoxia Hallmark *A) Comparison of siNEG 0.5% O2 Vs siNEG 20% O2 at 24h and 48h; B) comparison of siTRAP1 vs siNEG under normoxia and hypoxia at both time points.*

Statistical analysis revealed also a role of TRAP1 in controlling the activation of mTORC1 signaling pathway in response to hypoxia. Indeed, mTORC1 is a protein complex that functions as a nutrient/energy/redox sensor and plays a central role in sensing environmental conditions and regulating nearly all aspects of metabolism (Saxton, et al., 2017). As for the hypoxia hallmark, mTORC1 signaling pathway also showed an upregulation in response to hypoxia in control cells with conserved expression of TRAP1 at 24h (nes= 1.63; pvalue=0.001) and at 48h (nes= 1.69; pvalue=0.001). By contrast, TRAP1-silenced cell lines exposed to hypoxia showed a consistent dowregulation of mTORC1 signaling both at 24h (nes=-1.42; pvalue= 0.001) and 48h (nes=-1.68; pvalue=0.001) **Table 6 A-B**.

A

Into siNEG				24hrs 0,5%O2 Vs 20% O2					48hrs 0,5%O2 Vs 20% O2				
Row.Names	adj.Fisher	Fisher	N.pvalue	Es	pvalue	Nes	N.leading	N.geneSet	Es	pvalue	Nes	N.leading	N.geneSet
HALLMARK_MTORC1_SIGNALING	9,48611E-	2,65611E-07	3	0,493737	0,001	1,631713	50	189	0,468456	0,001	1,694322	45	189

B

siTRAP1 Vs siNEG				24hrs 20%O2					24hrs 0,5%O2				
Row.Names	adj.Fisher	Fisher	N.pvalue	Es	pvalue	Nes	N.leading	N.geneSet	Es	pvalue	Nes	N.leading	N.geneSet
HALLMARK_MTORC1_SIGNALING	3,90788E-	3,90788E-07	3	0,393925	0,0005	1,66556	32	189	-0,39091	0,005	-1,42234	34	189

48hrs 20%O2					48hrs 0,5%O2				
Es	pvalue	Nes	N.leading	N.geneSet	Es	pvalue	Nes	N.leading	N.geneSet
0,2836393	0,0725	1,153806	35	189	-0,42241	0,001	-1,68802	58	189

Table 6 – mTORC1 signaling Hallmark *A) Comparison of siNEG 0.5% O2 Vs siNEG 20% O2 (siNEG) at 24h and 48h; B) comparison of siTRAP1 vs siNEG under normoxia and hypoxia at both time points.*

Alltogether, these data suggest that TRAP1 is a key determinant of cell response to hypoxia and its expression is crucial in eliciting the activation of survival and metabolic pathways in cell exposed to oxygen deprivation conditions.

8.7 TRAP1 Regulates Response to Hypoxia in Organoids

During my internship at the Netherlands Cancer Institute in Amsterdam, I had the opportunity to work with a 3D-model of human colorectal cancer organoids, and create an shTRAP1 organoid model. Four different lentiviral plasmids were tested and validated to obtain the stable knocking down of TRAP1. shTRAP1 constructs were obtained from NKI's Robotics and Screening Center facility. Tests were performed first on HCT116 colorectal cell lines and HEK293T cells. With the exception of shTRAP1 #4, qRT-PCR results showed a high silencing efficiency of TRAP1 by lentiviruses shTRAP1 #1 (85% and 75% in HEK293T and HCT116 cells respectively), shTRAP1 #2 (70% and 60% in HEK293T and HCT116 cells respectively) and shTRAP1 #3 (95% and 70% in HEK293T and HCT116 cells respectively) **Figure 23 A-B**

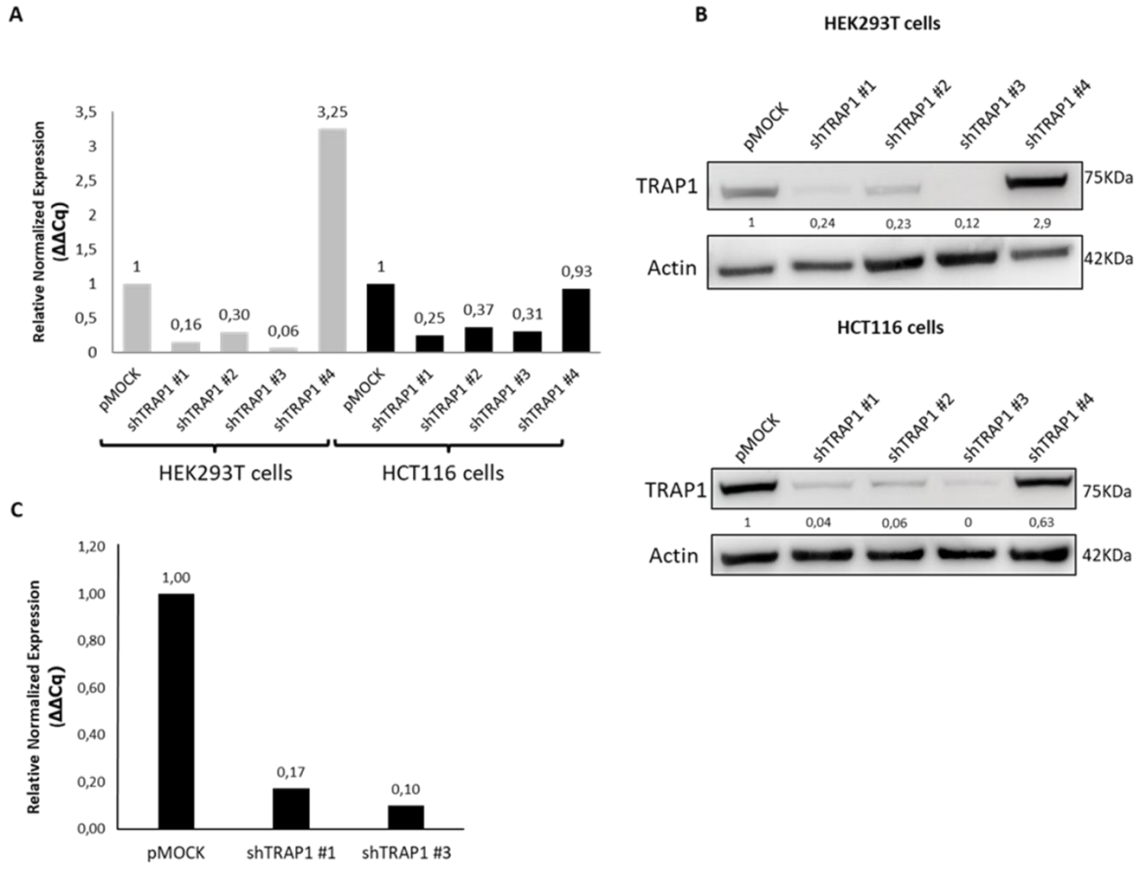
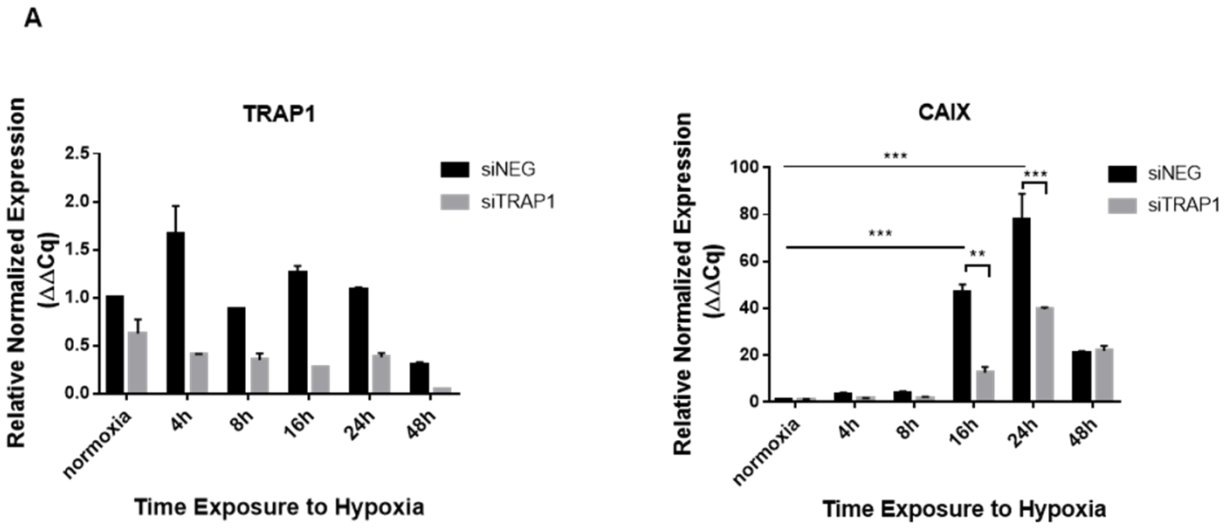


Figure 23 – Lentiviruses efficiency on HEK293T cells, HCT116 cells and P9T organoids *(A) TRAP1 relative qRT-PCR analysis was performed in respectively HEK293T cells and HCT116 cells transduced with 4 different shTRAP1 lentiviruses (# 1, # 2, # 3, # 4) and control vector pMOCK. (B) Immublot analysis performed on the same samples described in A. (C) Efficiency of transduction of P9T organoids with shTRAP1 #1 and shTRAP1 #3 lentiviruses. β-actin was used as housekeeping gene.*

The shTRAP #1 and #3 lentiviral plasmids were selected for the transduction of P9T organoids previously selected based on the high expression level of TRAP1 (data not shown). Lentiviruses shTRAP #1 and shTRAP1#3 were shown to have excellent knockingdown activity in organoids with respectively 80% and 90% of mRNA downregulation **Figure 21C**. Based on the rationale that the growth of organoids within a 3D matrix allows the spontaneous generation of the hypoxic nucleus observed in the tumor (Silva-Almeida, et al., 2019), this model was used to evaluate the hypoxic response respect to TRAP1 expression levels. The P9T shTRAP1#1 organoids (faster in growth) were used

to evaluate the hypoxic response dependent on TRAP1 expression levels. A time couse experiment was set up in response to normoxia and hypoxia exposure: pMOCK control and TRAP1-silenced P9T organoids were incubated for 4h, 8h,16h, 24h and 48h at 0.5 and 20% O2 and analyzed by Real-Time PCR for HIF-1α inducible genes (the same previously evaluated in HCT116 cells). CAIX was strongly upregulated after 16h and 24h of hypoxia in control siNEG organoids exposed to 0.5% O2 (p-value <0.001), while its upregulation was significantly reduced at 16h (p-value <0.01), and at 24h (p-value <0.001) in P9T siTRAP1#1, this indicating that TRAP1 modulated cell response to hypoxia in a 3D model **Figure 24A**.



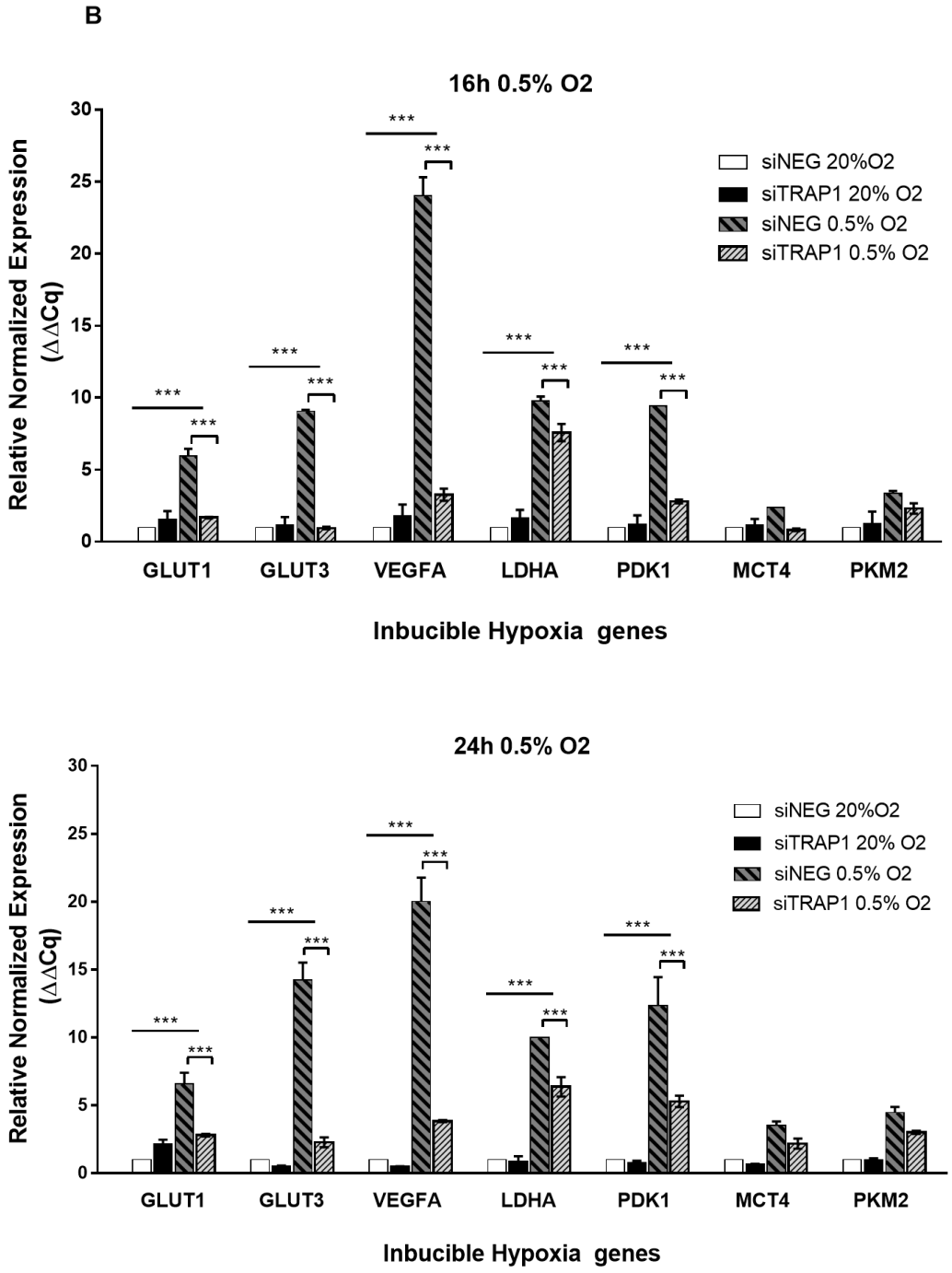


Figure 24 – Time course of oxigen deprivation in control and siTRAP1 #1 P9T organoids. *(A) Organoids were exposed to hypoxia (0.5% O2) or normoxia (20% O2) for 4h, 8h 16h, 24h and 48h. Relative mRNA expression levels for TRAP1 and CAIX at all time points. (B) qRT-PCR was performed using primers for HIF-1α inducible gene, GLUT1, GLUT3 VEGFa, LDHA, PDK1, MCT4 and PKM2, at 16h and 24h time points. Ordinate values are based on standardization of*

target gene expression to that of α-Tubulin and β-actin. Statistical significance is indicated with (***) p< 0.001 (ns= not significant) comparing siNEG 0.5%O2 Vs siNEG 20%O2, and siTRAP1 0.5% O2 Vs siNEG 0.5% O2.

Then, the modulation of inducible HIF-1α genes was assessed only at these two time points. MCT4 and PKM2 did not show any significant modulation in response to oxygen deprivation and the presence/absence of TRAP1 at all times points. By contrast, all other genes (i.e., GLUT1, GLUT3, VEGFA, LDHA and PDK1) showed a significant upregulation when exposed to hypoxia at 16h and 24h (p-value <0.001), and a reduced upregulation in the same conditions of oxygen deprivation when TRAP1 was silenced, p<0.001 **Figure 24B**.

9 DISCUSSION

Cancer cells are typically subjected to profound metabolic alterations, including the "Warburg effect" (Warburg, 1956), where in most cancer cells predominantly produce energy by a high rate of aerobic glycolysis. Cells constantly adjust their metabolic state in response to extracellular signals and nutrient availability to meet their demand for energy and metabolic building blocks. Metabolic reprogramming is now considered a hallmark of tumorigenesis (Galluzzi, et al., 2013). Recent studies suggest that targeting cancer cell energy metabolism might be a new and very effective therapeutic approach for selective ablation of malignancies (Gogvadze, et al., 2009; Morin, et al., 2014; Rossignol, 2015)

In this context, TRAP1 is a mitochondrial molecular chaperone known for its ability to regulate the adaptive response of cancer cells to adverse environmental condition such as oxidative stress, ER stress, drug therapies, apoptosis, modulating, directly or through its protein clients, numerous pathways (Chae, et al., 2012; Maddalena, et al., 2013). TRAP1 and its network of client proteins are upregulated in colorectal carcinoma in 50-60% of cases and are predictive of poor prognosis (Maddalena F, *et al*, 2017). Moreover, additional evidence supports the role of TRAP1 in favoring glycolytic metabolism (Soichiro Yoshida *et al*, 2013), suggesting that TRAP1 expression levels influence the response to bioenergetic requirements of cancer cells in a context-dependent manner (Matassa DS *et al*, 2018). Indeed, many human malignant malignancies (colorectal, breast, nasopharyngeal and pulmonary carcinomas), with high TRAP1 expression, are characterized by a predominantly glycolytic metabolic profile, while other tumors (ovarian, renal and cervical carcinomas) with low TRAP1 expression levels are characterized by oxidative metabolism.

Glycolytic metabolism in hypoxic tumors is mainly associated to the capacity of the transcriptional factor HIF-1α to intervene on the expression of target genes as glucose transporter (GLUT) isoform 1 (GLUT1) and 3 (GLUT3) (Semenza, 2010), lactate dehydrogenase A (LDHA), as well as the lactate-extruding enzyme monocarboxylate

transporter 4 (MCT4) (Pouyssegur et al., 2006; Semenza, 2007) CAIX, (Fang JS *et al,*2008), PDK1 (Kim JW *et al,* 2006) and PKM2 (S. Mazurek m, 2011). The shift from oxidative to glycolytic metabolism allows maintenance of redox homeostasis and cell survival under conditions of prolonged hypoxia. Many metabolic abnormalities in cancer cells increase the activity of HIF-1α.

Herein we tested the hypothesis that TRAP1 favors metabolic adaptation in human CRC in the specific hypoxia context. Our data suggest that i) high TRAP1 expression correlates with enhancement of Warburg metabolism in human CRC samples and HCT116 cell line, ii) TRAP1 acts as a HIF-1α stabilizer, iii) under hypoxic conditions, TRAP1 influences the ability of HIF-1α to activate the metabolic cellular adaptation, according to oncogenic program in HCT116 cells and in patients-derived organoids.

Our results suggest that TRAP1 is an important regulator of glycolysis, both *in vitro* and *in vivo*. Data on glucose uptake and expression of GLUT1 transporter protein indicate that the molecular chaperone promotes carbohydrate internalization, probably increasing the number of carriers exposed on the cell membrane. Furthermore, cells expressing TRAP1 produce higher amount of lactate, an indication of a more consistent glycolytic metabolism compared to the TRAP1 silenced cells. This process in more pronounced under hypoxia, which represents by itself a condition that favors glycolytic metabolism.

Furthermore, all metabolic genes regulated by HIF-1α are partially blocked when TRAP1 is downregulated, with a reduction in expression levels of all transcripts for long-term exposure to hypoxia. In addition, the protein expression shows a direct correlation between TRAP1 expression levels, HIF-1α stabilization and protein modulation. Since TRAP1 downregulation prevents HIF-1α stabilization, which results in the reduction of HIF-1α related metabolic circuits, these data allow us to hypothesize a deep correlation between TRAP1, HIF-1α and metabolic rewiring in colorectal cancer, demonstrating how the molecular chaperone TRAP1, an oncogene in colorectal cancer, promotes a glycolytic phenotype and guides the activation of HIF-1α to promote tumor progression.

A new role of TRAP1 is outlined in the adaptation of cancer cells to adverse conditions, expanding the chaperone's actions scenario as a stress protein and candidate it as a target protein for the development of targeted molecular therapies in colorectal cancer.

The gene expression profile analysis allowed also the identification of pathways and biofunctions that underlie this correlation, in an attempt to discover its mechanism. Among others, mTORC1 was identified as potentially involved in TRAP1 reposnse to hypoxia. Furthermore, the current availability of an organized and complex system such as the 3D organoids model, will be of great help to understand the metabolic dynamics that are established between different cell types present *in vivo*, where the metabolic reprogramming is regulated by the interplay between cancer cells and neighbour cells.

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